Vol. XLIV No. 3

March 1955

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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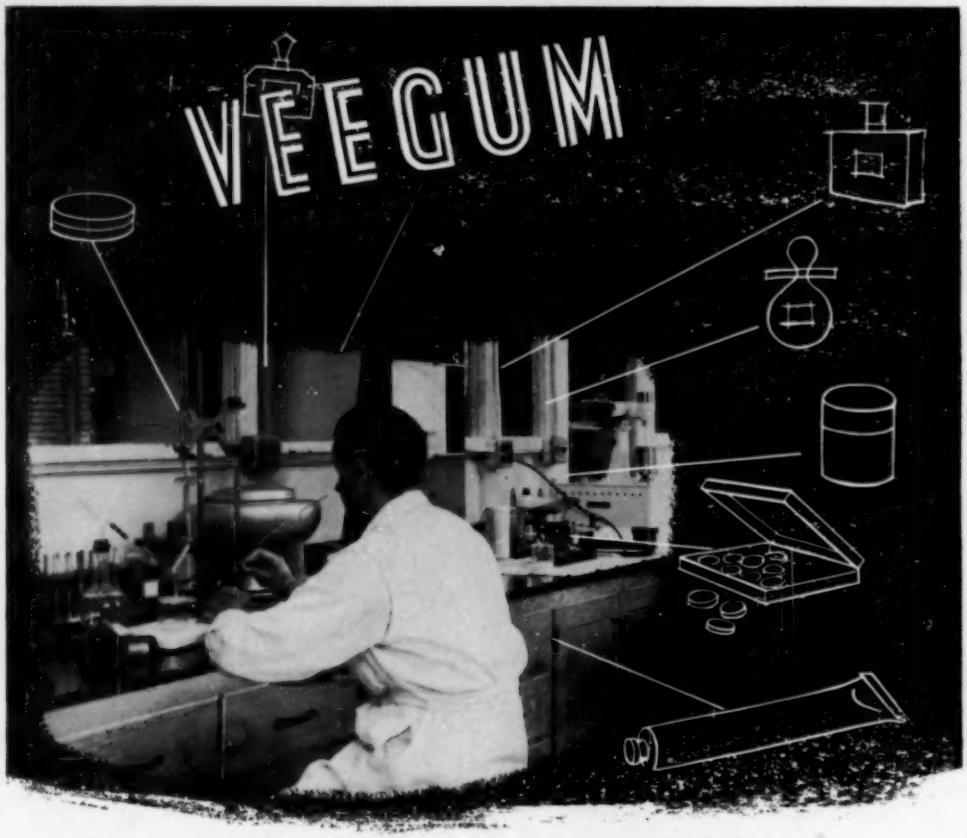

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The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

Editorial and Executive Offices—2215 Constitution Avenue, N.W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition and Scientific Edition combined: United States, Canada, Pan America, Spain, and Philippines, $10.00; other foreign, $12.00.

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CONVENTION ANNOUNCEMENT

The 102nd convention of the American Pharmaceutical Association will be held at Miami Beach, Florida during the week of May 1, 1955. The fact that this convention will be held in one of America's outstanding seashore resorts at a time when it is possible to combine a vacation with the customary professional activities of the American Pharmaceutical Association and its affiliated and related organizations, will draw an unusually large attendance.

No single hotel will accommodate all who are planning to attend this convention. However, there are many splendid hotels within close proximity of each other and there will be no dearth of luxury accommodations at greatly reduced vacation resort prices.

The convention headquarters will be the Fontainebleau Hotel which has been completed only recently but which offers superb facilities. It will not be possible to hold all of the smaller group meetings at this hotel, but the major sessions of the convention can be accommodated there.

It will not be possible to house all who are expecting to attend the convention at the Fontainebleau but a majority of our members will find accommodations there. All of the other hotels listed on the form on the opposite page are outstanding in their facilities and every ocean front hotel provides bathing facilities including ocean bathing as well as swimming pool accommodations directly from the rooms in the hotel.

Such meetings as cannot be accommodated at the Fontainebleau Hotel will be held in the adjoining Empress Hotel. Special arrangements have been made for meetings and hotel accommodations at the Roney Plaza Hotel for the Student Section of the A.PH.A. and special student rates are being provided there.

The tentative program for the meetings of the A.PH.A. and its Sections and for the affiliated and related groups will be available in the March issue of the Practical Pharmacy Edition of THIS JOURNAL which will reach our members very shortly after this issue of the Scientific Edition.

Each member will receive also, by direct mail, a further outline of the program and a form on which to apply for room accommodations through the Housing Bureau for the Miami Beach convention.

Neither the ASSOCIATION office nor the individual hotels can undertake to make hotel reservations as this task has been assigned to the Housing Bureau which has been set up in the name of the Association at Miami Beach, Florida. *Please write only to the Housing Bureau for accommodations*, using hotel reservation form on the opposite page or the form which will reach you by mail. The form should be filled in completely, giving all information requested, otherwise it may be necessary to return it for completion and thus delay assignment of accommodations.

Robert P. Fischelis, *Secretary*
AMERICAN PHARMACEUTICAL ASSOCIATION

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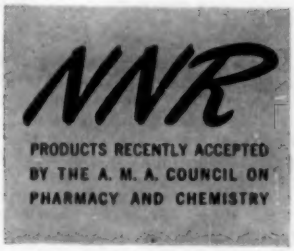

VIOMYCIN SULFATE.—Vinactane Sulfate (Ciba). —Viocin Sulfate (Pfizer).—Viomycin sulfate is the salt of an antibiotic substance or substances produced by the growth of *Streptomyces puniceus* on suitable culture media. Viomycin is a strongly basic polypeptide. Its chemical structure is not yet known.

Actions and Uses.—Viomycin sulfate is an antituberculous agent effective against both streptomycin-sensitive and streptomycin-resistant strains of tubercle bacilli, and also against isoniazid-sensitive and isoniazid-resistant organisms. Viomycin is less active than streptomycin but more active than *p*-aminosalicylic acid. Drug-resistant strains of tubercle bacilli develop with viomycin as with streptomycin and isoniazid, but cross resistance apparently does not occur between viomycin and streptomycin or isoniazid. As with streptomycin, it is believed that the incidence and rate of emergence of strains resistant to viomycin may be minimized by the combined use of *p*-aminosalicylic acid.

Viomycin sulfate is useful as an adjunct in the treatment of progressive, exudative, hematogenous lesions, and the pneumonic type of pulmonary tuberculosis; cutaneous tuberculous lesions and fistulas; tuberculous lymphadenitis; tracheobronchial and laryngeal tuberculosis; alimentary, peritoneal, genitourinary, meningeal, miliary, and bone and joint tuberculosis; and postoperatively in thoracic surgery on tuberculous patients. It is useful prophylactically in operations such as lobectomy and pneumonectomy for pulmonary tuberculosis. Because of its potential toxicity, viomycin is not recommended for use in the treatment of minimal or primary pulmonary tuberculosis when routine treatment or other forms of drug therapy is effective, such as streptomycin, isoniazid, or *p*-aminosalicylic acid, singly or in combination. Viomycin thus should be restricted for use in the treatment of patients unable to tolerate these drugs or patients with tuberculous disease caused by organisms resistant to them. Like other antituberculous drugs, viomycin is of relatively little value in the therapy of extensive caseous or fibrotic lesions.

Viomycin sulfate is absorbed readily following parenteral administration by the usual routes and is excreted largely by the kidneys. It should be administered by deep intramuscular injection; oral administration is ineffective as a means of obtaining chemotherapeutic blood levels. Only a small portion of the drug present in the blood seems to diffuse into the spinal fluid or the pleural and peritoneal cavities. More investigation is necessary to establish the usefulness of the intrathecal route, in conjunction with intramuscular injection, for the therapy of tuberculous meningitis. Intravenous administration must be avoided because it involves greater danger of toxic reactions.

Viomycin sulfate is a potentially toxic agent, and the manifestations of toxicity are related chiefly to dosage. Preexisting renal damage may be aggravated and, in patients with impairment of renal function, serious reactions may result from interference with the normal excretion of the drug. These patients should receive lower dosages and be carefully observed for signs of toxicity. Such signs, which should be looked for in all patients receiving the drug, include allergic reactions, renal irritation (albuminuria or cells or casts in the urine), eosinophilia, edema or fluid retention as evidenced by increased weight, electrolyte disturbance, abnormal renal function, dizziness, electrocardiographic abnormalities, and partial loss of hearing. Skin eruptions frequently may be controlled with antihistamine drugs. Audiometric testing should be done prior to and at regular intervals during treatment to detect any hearing impairment that may occur. It has not been determined whether or not auditory impairment is permanent. Disturbances in the serum electrolyte pattern may be alleviated readily by the administration of supplemental potassium chloride. The afore-mentioned toxic reactions are unlikely to occur with any degree of frequency or severity when recommended dosages are administered.

Dosage.—Viomycin sulfate is administered by intramuscular injection, preferably into the gluteal, thigh, or deltoid muscles. It is important to rotate the site of injection with each dose, and injection should be made slowly. Dosage is expressed in terms of the equivalent weight of viomycin base. The drug is diluted with either water for injection or isotonic sodium chloride solution to make concentrations not exceeding 0.5 Gm. per cubic centimeter. In the dry form it may be stored at room temperature for 24 months without appreciable loss of potency. Solutions may be stored at room temperature under sterile conditions for one week without significant loss of potency, but it is recommended that they be stored in a refrigerator.

For most forms of tuberculosis, an intramuscular dose of 2 Gm. (given in two doses of 1 Gm. each 12 hours apart) every third day in recommended, either alone or in conjunction with *p*-aminosalicylic acid (12 Gm. daily by the oral route). At this dosage, therapy should be continued for at least four to six months, depending on the response of the lesion. In special instances, a daily dosage not to exceed 2 Gm. (in divided doses) for a period of not more than one month may be administered if facilities are available for repeated measurement of serum electrolytes, renal and hepatic function, and audiometric changes. In the presence of impaired renal function, the dosage should be much less than 2 Gm., every third day, with very close observation of the patient for toxic manifestations. When the total daily dosage is more than 1 Gm., it must be administered in two equal divided doses separated by a 12-hour interval.

(Continued on VII)

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 200 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

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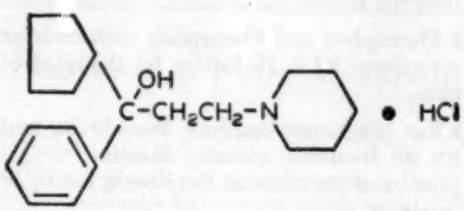
N.N.R. ..... *from page V*

Tests and Standards.—

Physical properties: Viomycin sulfate is a white or slightly yellow, odorless powder. It decomposes between 225 and 265°. It is very soluble in water and very slightly soluble in alcohol, methanol, ether, benzene, and in chloroform. It is moderately hygroscopic. The pH of a 10% solution is 4.5–7.0.

[For more information regarding this monograph see *J. Am. Med. Assoc.* **157**, 344(January 22, 1955).]

CYCRIMINE HYDROCHLORIDE.—Pagitane Hydrochloride (Lilly).—$C_{19}H_{29}NO.HCl$.—M.W. 323.90.—α-Cyclopentyl-α-phenyl-1-piperidinepropanol hydrochloride.—Cyclopentyl-phenyl-3-(1-piperidyl)-1-propanol hydrochloride.—The structural formula of cycrimine hydrochloride may be represented as follows:



Actions and Uses.—Cycrimine hydrochloride, an aminopropanol chemically related to trihexyphenidyl hydrochloride, is an atropine-like antispasmodic agent that constitutes a useful addition to the class of drugs employed in the symptomatic treatment of Parkinson's disease (paralysis agitans). Pharmacological studies in animals indicate that cycrimine hydrochloride has parasympatholytic action and a direct antispasmodic effect on smooth muscle, but it produces little or no antihistaminic action. Compared with atropine, it has about one-half as much spasmolytic effect and about one-tenth as much antisialogogue effect. Likewise, it produces much less cardiovagal inhibition. The drug also has both mydriatic and ophthalmic anesthetic properties.

Cycrimine hydrochloride frequently is effective in the treatment of all three types of paralysis agitans: postencephalitic, arteriosclerotic, and idiopathic. The drug is more universally effective when the disease is based on a postencephalitic cause, and less often effective when the condition is caused by arteriosclerotic changes.

In experimental animals cycrimine hydrochloride is slightly more toxic than atropine. Its use should be avoided in conditions in which inhibition of the parasympathetic nervous system is undesirable. For example, it should probably not be administered in the presence of glaucoma and should be used with caution in the presence of tachycardia or any tendency toward urinary retention.

Clinically, the incidence and degree of side-effects is chiefly a result of dosage. Side-effects commonly observed include dryness of the mouth, blurring of vision, epigastric distress, and transient nausea with anorexia. Since these effects may subside with continued therapy, discontinuance of the drug is not ordinarily required. Epigastric distress often can be overcome by administering medication with meals or with milk. More serious side-effects, such as vertigo or disorientation, make it imperative to reduce the dosage or discontinue therapy entirely.

Dosage.—Cycrimine hydrochloride is administered orally, preferably with meals. In general, patients with postencephalitic paralysis agitans tolerate larger doses than those having the arteriosclerotic or idiopathic types of the disease; elderly patients, especially of the arteriosclerotic type, likewise, do not tolerate large single doses. The dosage should be individualized, and, when tolerance is poor, adequate total daily dosage often can be achieved with frequent administration of small doses.

For postencephalitic paralysis agitans, an average initial total daily dosage of 15 mg. (5 mg. three times daily) is suggested, but this is subject to individualization between a daily minimum of 10 mg. (2.5 mg. four times daily) and a maximum of 45 mg. (5 mg. every 2 hours, covering 16 waking hours). For arteriosclerotic and idiopathic types of the disease, an average initial total daily dosage of 7.5 mg. (2.5 mg. three times daily) is suggested, but this is also subject to adjustment between a daily minimum of 5 mg. (1.25 mg. four times daily) and a maximum of 20 mg. (5 mg. four times daily). Should the suggested initial dosage produce side-effects, it should be gradually reduced until an optimum response is achieved with little or no side-effects. When the initial dosage fails to induce a therapeutic response, it should be gradually increased to a point that is optimal from the standpoint of response and tolerance to side-effects.

Tests and Standards.—

Physical Properties: Cycrimine hydrochloride is a white, odorless, bitter solid, m.p. 241–244° (decompn.). It is practically insoluble in benzene and in ether. The approximate amounts that dissolve at 25° in the following solvents to form 100 ml. of solution are: 2 Gm. in alcohol, 3 Gm. in chloroform, and 0.6 Gm. in water. The pH of a 0.5% solution is 4.9–5.4.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **157**, 510(February 5, 1955).]

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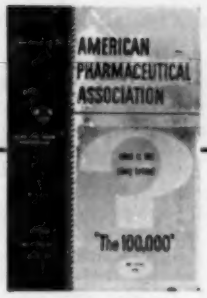

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *MARCH, 1955* — NUMBER 3 — CONSECUTIVE NO. 6

Methods for the Determination of Protoveratrine*

By ERNEST W. GRANT and EDWARD E. KENNEDY

Infrared and titrimetric procedures for the evaluation of protoveratrine mixtures are reported which are not specific for either protoveratrine A or protoveratrine B. A method for the quantitative estimation of protoveratrine A and protoveratrine B is described in which the alkaloids are separated by paper partition chromatography, eluted, and determined colorimetrically.

PROTOVERATRINE was first isolated from *Veratrum viride* by Salzberger (1) in 1890 and Edens (2) reported on its pharmacology in 1892. The current availability of the drug is due to the development of extraction methods by Poethke (3) and by Craig and Jacobs (4). Protoveratrine has since been subjected to extensive clinical investigation and its value in the treatment of hypertension was established by Meilman and Krayer (5) in 1949. Since then protoveratrine has proved to be a valuable hypotensive agent because of its high and uniform potency.

In 1953, Nash and Brooker (6) showed that the protoveratrine prepared by the original method of Craig and Jacobs actually consisted of two components—protoveratrine A, $C_{41}H_{69}O_{14}N$, and protoveratrine B, $C_{41}H_{69}O_{15}N$. [Protoveratrine B has also been reported in the literature as oxyprotoveratrine, neoprotoveratrine (7) and veratetrine (8).] The two alkaloids have very similar chemical and physical properties and therefore usually require rather long and involved methods of separation. Inasmuch as pharmacological studies (9) have indicated that the action of the two alkaloids is similar, separation is not attempted and normally the commercially available protoveratrine is a mixture of approximately equal parts of protoveratrine A and protoveratrine B. As a result, chemical control of protoveratrine involves both the estimation of the total protoveratrine in the mixture and the determination of the relative amounts of its components. The purpose of this paper is to present briefly some of the control procedures and to describe a paper chromatographic method for the evaluation of these mixtures. The method may also be applied to mixtures of veratrum alkaloids containing other alkaloids in addition to protoveratrine.

* Received August 27, 1954 from the Eli Lilly & Company, Indianapolis, Ind.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.

EXPERIMENTAL

Determination of Protoveratrine Mixture.—The protoveratrine mixture may be estimated quantitatively by titrimetric or infrared procedures. In the titrimetric method, approximately 100 mg. of the alkaloid is dissolved in 25 cc. of 0.02 *N* sulfuric acid, and the residual acid titrated with 0.02 *N* sodium hydroxide using bromcresol green as the indicator. The alkaloid also may be determined by nonaqueous titration techniques, in which the alkaloid is dissolved in glacial acetic acid and chloroform and titrated with perchloric acid in dioxane. The analytical factor is based on an average of the molecular weights of the two components. These titrimetric methods are obviously not specific and serve only to measure the total alkaloidal bases.

Protoveratrines A and B are ester alkaloids and therefore show a very strong infrared absorption band at approximately 5.8 μ due to the carbonyl linkage. The intensity of this carbonyl band may be used for the quantitative evaluation of protoveratrine and protoveratrine preparations. The

absorption of a solution containing approximately 5 mg./cc., at 5.8 μ and in 0.7 mm. rock salt cells, is compared with that of a standard. The standard is composed of equal parts of protoveratrine A and protoveratrine B.

Tablets and ampuls of protoveratrine may be assayed by this infrared procedure. The alkaloid is extracted, dissolved in chloroform, and the absorption compared with an amount of protoveratrine standard equivalent to the theoretical content of the preparation.

The infrared procedure also may be used in stability studies, to determine the deterioration of protoveratrine preparations. A decrease in the absorption in the carbonyl region indicates hydrolysis of the protoveratrine with a subsequent loss in hypotensive potency. Papineau-Couture and Burley (10) have shown that the carbonyl band always accompanies hypotensive activity and used this property to evaluate quantitatively the hypotensive activity of extracts of *Veratrum viride*.

Determination of Protoveratrine A and Protoveratrine B.—Because of the marked similarity in their chemical and physical properties, the determination of protoveratrine A and protoveratrine B usually involves complex analytical procedures. There are slight differences in the infrared spectra of protoveratrines A and B between 8 and 10 microns. These differences can be utilized in a quantitative fashion by establishing the absorption ratios, 8.52 μ/8.68 μ or 9.18 μ/9.60 μ (11). By plotting ratio vs. per cent composition of known mixtures, it is possible to obtain a calibration curve suitable for quantitative use. However, the precision and accuracy of this method are greatly affected by the presence of small amounts of other veratrum alkaloids or impurities and its use is limited to mixtures containing only protoveratrine A and protoveratrine B.

Other methods involve the separation of the components by countercurrent distribution or chromatography. Countercurrent distribution between chloroform, water, acetic acid (6) or between benzene and 2 *M* acetate buffer (*p*H 5.5) (7) may be used to separate the two components. However, small amounts of contaminating alkaloids interfere and at least a 24-plate distribution is usually required for the satisfactory resolution of the mixture. The equipment involved, the time and labor required, and the size of sample preclude the use of this method as a routine analytical procedure.

The components may be separated by passing the mixture through an alumina column. However, this is also a time-consuming procedure and recoveries are not sufficiently quantitative for control use. A method based on paper partition chromatography is presented below, which avoids many of the disadvantages of the methods described above. The proposed technique is simple in operation, requires no complex equipment, and results are reproducible. The precision is not affected by the presence of other veratrum alkaloids and the method may be used for the determination of protoveratrine A and protoveratrine B in mixtures of veratrum alkaloids.

Reagents and Apparatus:

Developing solvent—Benzene saturated with formamide.

Dragendorff's reagent

Solution I
0.85 Gm. bismuth subnitrate
10.0 cc. acetic acid
40.0 cc. distilled water

Solution II
8.0 Gm. potassium iodide
20.0 cc. distilled water

Add 5 cc. of each solution to 20 cc. of acetic acid and dilute with 100 cc. distilled water.

Chromatographic apparatus—A 12 x 24-inch jar equipped for the descending technique.

Paper—Whatman No. 1.

Beckman Model DU Spectrophotometer.

PROCEDURE

Fold and crease the filter strips (1/2 x 22 in.) in the usual manner for descending chromatography and dip them in an acetone-formamide (3:1) solution. Place the filter strips between two sheets of blotting paper to remove any excess solution. Apply measured amounts of the sample and measured amounts of the standard in chloroform to the treated strips and allow them to air-dry for five minutes. Transfer these strips, as well as a blank-treated strip, to the chromatographic jar and allow the chromatograms to develop for eighteen to twenty hours. During this period, the developing solvent will travel off the end of the filter strip.

Remove the strips from the jar, air-dry for fifteen minutes and then dry in a 50° oven for one to two hours. Prepare guide strips by spraying one of the standard strips and one of the sample strips with the Dragendorff's reagent. Remove any excess reagent by blotting. The alkaloids will appear as reddish-orange spots. Protoveratrine B will show little or no travel while protoveratrine A will travel from 6 to 8 inches.

Mark the remaining standard and sample strips, using the guide strips to locate accurately the position of the alkaloids. Cut the protoveratrine A and protoveratrine B sections from each strip and place the segments into separate glass-stoppered test tubes. (The individual segments may be cut into 3–4 smaller pieces to aid in the extraction of the alkaloid.) Add 15 cc. of ethanol, stopper the tubes and shake mechanically for thirty to forty-five minutes. Filter each eluate into a 50-cc. beaker. Wash the segments with several small portions of ethanol, filter these washings through the same filter and combine them with the original filtrate.

Evaporate the eluates to dryness on a steam bath with the aid of a current of air and dry the residues *in vacuo* at 50° for one hour. To each residue add exactly 5 cc. of sulfuric acid and allow the color to develop for eighteen hours at room temperature. Determine the absorbancies at 540 mμ, using 1-cm. matched cells and sulfuric acid as the blank. The relative protoveratrine A and protoveratrine B content is determined by comparing the absorbancies of the sample, corrected for the blank, with the corrected absorbancies of the standard.

DISCUSSION

The benzene-formamide system has been used by several workers for the separation of alkaloids and other natural products (12, 13). Satisfactory sepa-

ration of the protoveratrine components was obtained with this system and the protoveratrine A zone was less diffuse than with other systems studied. The relatively narrow (1/2-in.) filter strip proved to be more satisfactory than wider strips. Although the protoveratrine A zone tended to be slightly more compact in wider strips, the area of each individual segment was so large that quantitative elution with small amounts of ethanol was difficult.

Preliminary studies indicated that a sample containing 300 μg. of each component was the most convenient for quantitative estimation. Separation and elution of the components were satisfactory and the reaction with sulfuric acid yielded a color of sufficient intensity. Accordingly, separate chloroform solutions of protoveratrine A and protoveratrine B were prepared as standards and volumes containing 300 μg. of each were transferred to the standard filter strip by means of a micro-pipet. The mixture to be evaluated was also dissolved in chloroform, and a volume containing 600 μg. of the mixture placed on the sample filter strip. In order to correct for any extraneous materials present in the paper or the reagents, a blank strip, treated in the same mammer as the other strips, was chromatographed at the same time. Lengths of the blank strip equal to the lengths of the protoveratrine A and B zones were extracted and the necessary blank corrections determined. Usually 1 blank, 3 standard, and 3 sample chromatograms were developed for each assay.

It was necessary to filter the eluates to remove loose filter fibers which would be charred by the sulfuric acid and interfere with the colorimetric determination. Vacuum filtration was rapidly accomplished with a micro bell jar and a small Büchner funnel.

There are obvious disadvantages to using a sulfuric acid color reaction in quantitative procedures. However, this reaction appeared to be the only satisfactory one for evaluating the small quantities involved. At the end of eighteen hours, the colors produced were relatively stable and sufficiently intense for accurate measurement. The absorption maximum of both the protoveratrine A and the protoveratrine B reaction product was at approximately 540 mμ, with an isobestic point at 492 mμ. Spectrophotometric readings were made at 540 mμ since the blank interference was considerably lower at this wavelength.

The data in Table I shows reasonably good linearity among different concentrations of the components. However, results were not consistently reproducible due to variations inherent in the chromatographic process; thus it was not possible to utilize a permanent calibration curve. Instead of preparing a new calibration curve for each determination, a standard mixture composed of 300 μg. of each component was chromatographed at the same time as the unknown mixture. The specific absorbance ($A^{1\ \mu g/5cc.}_{1\ cm.}$) value obtained from the standards was used in determining the relative protoveratrine A and B content of the unknown mixture.

TABLE II.—CHROMATOGRAPHIC DETERMINATION OF PROTOVERATRINE MIXTURES

Sample No.	Composition of Mixt.	Recovered μg.	Each Component Recovered, %	Relative % of Each Component in Total Recovery
1	150 μg. A	158	105.1	35.9
	300 μg. B	282	94.0	64.1
		440		
2	150 μg. A	154	102.6	35.1
	300 μg. B	285	95.0	64.9
		439		
3	150 μg. A	147	98.0	33.3
	300 μg. B	294	98.0	66.7
		441		
4	300 μg. A	317	105.7	50.2
	300 μg. B	315	105.0	49.8
		632		
5	300 μg. A	323	107.6	51.1
	300 μg. B	310	103.3	48.9
		633		
6	300 μg. A	332	110.6	51.9
	300 μg. B	308	102.6	48.1
		640		
7	300 μg. A	305	101.6	69.3
	150 μg. B	135	90 0	30.7
		440		
8	300 μg. A	309	103.0	68.2
	150 μg. B	144	96.0	31.8
		453		
9	300 μg. A	301	100.3	66.3
	150 μg. B	153	101.9	33.7
		454		

TABLE I.—REPRODUCIBILITY AND LINEARITY OF PROPOSED CHROMATOGRAPHIC METHOD

Protoveratrine A			Protoveratrine B		
Sample	A (Corrected) at 540 mμ	$A^{1\ \mu g./5\ cc.}_{1\ cm.}$	Sample	A (Corrected) at 540 mμ	$A^{1\ \mu g./5\ cc.}_{1\ cm.}$
150 μg.	0.273	0.00182	150 μg.	0.246	0.00164
150 μg.	0.266	0.00177	150 μg.	0.240	0.00160
300 μg.	0.537	0.00179	300 μg.	0.477	0.00159
300 μg.	0.550	0.00183	300 μg.	0.486	0.00162
450 μg.	0.820	0.00182	450 μg.	0.732	0.00163
450 μg.	0.798	0.00177	450 μg.	0.720	0.00160
	Av.	0.00180		Av.	0.00162

RESULTS

Table I demonstrates the reproducibility of the color reaction and the linearity obtained with varying concentrations.

The chromatographic method was applied to the evaluation of mixtures of known composition and the results outlined in Table II. These results show a consistent relative recovery of the individual components and the accuracy of the method appears comparable with other quantitative chromatographic methods.

Table III gives data obtained from the evaluation of mixtures of veratrum alkaloids. These unknown mixtures represent the partially refined protoveratrine fractions obtained from *Veratrum album* drug and were therefore presumed to contain largely protoveratrines A and B. The duplicate results, being in close agreement on each sample, appear to be indicative of the actual quantities of the two major components.

TABLE III.—PROTOVERATRINE A AND B CONTENT OF MIXTURES OF VERATRUM ALKALOIDS

Sample No.	Determination No.	Recovered, μg.	% of A and B
I	1	298 μg. A	49.7 A
600 μg.		248 μg. B	41.2 B
	2	289 μg. A	48.2 A
		261 μg. B	43.5 B
II	1	241 μg. A	40.1 A
600 μg.		277 μg. B	46.2 B
	2	253 μg. A	42.2 A
		270 μg. B	45.0 B
III	1	228 μg. A	38.0 A
600 μg.		329 μg. B	54.8 B
	2	234 μg. A	39.0 A
		337 μg. B	56.2 B

SUMMARY

1. Methods for the evaluation of protoveratrine have been presented.

2. A paper chromatographic method for the determination of the relative amounts of protoveratrine A and protoveratrine B in protoveratrine mixtures has been described.

3. Data have been presented indicating the utility of the chromatographic method for determination of protoveratrines A and B in the presence of minor alkaloidal impurities.

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N-Bis(β-Chloroethyl)amino Acids and Related Compounds as Tumor-Growth Retarding Agents*

By MORIZO ISHIDATE, YOSHIO SAKURAI, and MICHIMASA IZUMI

A new series of N-oxide derivatives of nitrogen mustard were prepared and screened for possible physiological activity in retarding neoplastic growth. The nitrogen mustards, synthesized and screened, include N-bis(β-chloroethyl)glycine, -alanine, -taurine, and derivatives of these compounds.

NITROGEN MUSTARDS, viz., N-bis(β-chloroethyl)methylamine (HN2), are still interesting compounds as agents which retard neoplastic growth. As generally known, its practical application has been strictly limited, because of its high toxicity and its poor therapeutic index.

The chemotherapeutic mode of action of nitrogen mustards, which are cytotoxic alkylating agents in general, is a rather simple and nonspecific chemical reaction. The possible selectivity between normal and tumor cells seems to depend on the difference in sensitivity of the cells against such agents. Accordingly, the general toxicity of nitrogen mustards seems as a rule to be lower in those with weaker alkylating activity, with a concurrent decrease of effect against tumor cells. It is highly desirable that the selectivity of an agent between normal and tumor cells should be achieved by some chemical modification or by an increase of a certain physiological factor.

As the first step for this purpose a new series of N-oxide derivatives of nitrogen mustard were prepared in our laboratory and submitted to animal experiments (1, 2, 3). Some of these

* Received November 5, 1954, from the Pharmaceutical Institute, Medical Faculty, University of Tokyo, and Iatrochemical Institute of the Pharmacological Research Foundation, Tokyo.

The animal experiments were carried out by Dr. T. Yoshida (Professor, University of Tokyo) and his associates. The screening test refers to M. Ishidate, T. Yoshida, *et al.*, *Gann.*, **41**, 93(1950).

agents were shown to be much improved as compared to the original mustards.

As the next step, some new compounds of amino acids possessing the N-bis(β-chloroethyl) group have now been synthesized. After their chemical properties were examined they were submitted to animal experiments. The data covering their physical properties and the results of the screening test using sarcoma ascites (Yoshida) of rats are summarized in Tables I and II.

EXPERIMENTAL

N-Bis(β-hydroxyethyl)glycine was easily obtained by heating diethanolamine with chloroacetic acid in anhydrous alcohol. However, this was found to be rather wasteful for the material, because when it was chlorinated with thionyl chloride, morpholone derivatives were mainly produced. The following route was therefore found to be favorable in yield for the synthesis of N-bis(β-chloroethyl)glycine, -alanine, and the related compounds after Knoevenagel's procedure on dialkylaminoacetonitrile (4).

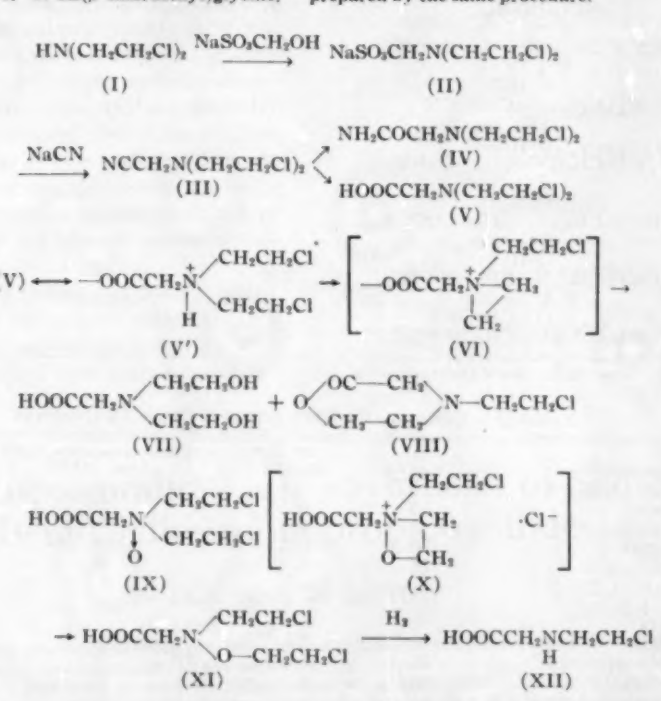
N-Bis(β-chloroethyl)taurine was easily prepared by the alkylation of taurine with ethylene oxide and the subsequent chlorination with thionyl chloride.

These N-bis(β-chloroethyl)amino acids are more hydrophilic and less toxic when compared with ordinary aliphatic nitrogen mustards and exhibit no vesicant action upon human skin. When their free carboxyl group was replaced forming an ester or amide, they exhibited toxicity as high as that of HN2.

The comparatively low toxicity of the free acids would be easily understood from the fact that the compounds are amphoteric (V′) and the reactivity of the chlorine atom is decreased by the formation of ammonium cation or by the apparent incapability of immonium formation. The velocity of Cl^- liberation, however, was unexpectedly much higher than that of HN2, and there was very little liberation of hydrogen ion. This indicated the existence of a cyclic onium ion as an intermediate step (VI), as in the case of HN2, and the like (5). The glycine derivative in a bicarbonate solution is finally hydrolyzed to N-bis(β-hydroxyethyl)glycine (VII) and partially transformed to the morpholone derivative (VIII).

It is worthy to note that, among the compounds prepared so far, N-bis(β-chloroethyl)alanine is the most interesting agent because it possesses a favorable chemotherapeutic index which implies a more selective action upon tumor cells (Table II).

According to the experience of our studies on the N-oxides of basic nitrogen mustards (2, 6) the N-oxides of the above acid nitrogen mustards were prepared by the same procedure.



For instance, N-bis(β-chloroethyl)glycine N-oxide (IX) gradually undergoes transformation into the hydroxylamine derivative (XI) when kept in a neutral aqueous solution at room temperature. The reduction potential of a fresh solution of the N-oxide measured by a polarographic method was found to be −1.013 V (vs. NCE) at pH 8.5, and with passage of time it decreased to about 0.421 V. In connection with the difference in the rate of liberation of the chlorine and hydrogen ions, the existence of an oximmonium ion of a four-membered ring (X) as an intermediate product of the compound (XI) is

indicated similarly to the case of the ordinary nitrogen mustard N-oxides (2, 6). The structure of the latter (XI) was well established by the isolation of β-chloroethyl glycine (XII) through the catalytic reduction of the compound (XI).

As shown in Table II, the comparative efficacy of

TABLE I.—MELTING POINTS OF SOME NEW COMPOUNDS OF AMINO ACIDS

Compound	m. p., °C.	m. p. of Picrate, °C.	m. p. of Picrylsulfonate, °C.
$(ClCH_2CH_2)_2N—CH_2—X$			
X: —CN·HCl	94	65	130
—$COOC_2H_5$·HCl	115	90	180
—$CONH_2$	70	137	178
—$CONH_2$·HCl	145	...	...
—$CONH_2$·H_2SO_4	192 (decompn.)	...	...
—COOH·HCl	76	...	220
$(ClCH_2CH_2)_2N—CH(CH_3)—X$			
X: —CN·HCl	108	97	...
—$CONH_2$	89	157	170 (decompn.)
—$CONH_2$·H_2SO_4	170	...	...
—$CONH_2$·HCl	190	...	...
—$COOC_2H_5$·HCl	...	...	158
—COOH·HCl	97	...	142 (decompn.)
$(ClCH_2CH_2)_2N—CH_2—COOH$	134	...	113
$(ClCH_2CH_2)_2N—CH_2—CN$ ↓O·HCl	...	115	...
$(ClCH_2CH_2)_2N—CH_2—CONH_2$ ↓O·HCl	156[a]	130	...
$(ClCH_2CH_2)_2N—CH_2—COOC_2H_5$ ↓O·HCl	52.5	109	...
$(ClCH_2CH_2)_2N—CH(CH_3)—COOH$ ↓O·HCl	110	...	...
$(ClCH_2CH_2)_2N—CH_2CH_2SO_3H$	179	...	...

[a] This sample was not in the state of analytical purity.

TABLE II.—COMPARATIVE EFFICACY OF N-OXIDE DERIVATIVES

Compounds	LD_{50}, mg./Kg.	MED,[a] mg./Kg.	Chemotherapeutic Index LD_{50}: MED[a]	C.E.[b]	P.L.[c]
$(ClCH_2CH_2)_2N—CH_2—X$					
X: —COOH	15	0.5	30	+[d]	+
—$COOC_2H_5$	3	0.1	30	+	−[d]
—$CONH_2$	0.8	0.1	8	+	+
—CN	75	5	15	+	
$(ClCH_2CH_2)_2N—CH_2—X$ ↓O					
X: —COOH	75	5	15	+	−
—$COOC_2H_5$	55	5	11	+	−
—$CONH_2$	30	1	30	+	
$(ClCH_2CH_2)_2N—CH(CH_3)—X$					
X: —COOH	30	0.05	600	+	+
—$CONH_2$	3	0.01	300	+	
$(ClCH_2CH_2)_2N—CH(CH_3)—COOH$ ↓O	55	5	11	+	
$(ClCH_2CH_2)_2N—CH_2CH_2SO_3H$	55	0.5	110	+	−

[a] MED: Minimum dose which can effect the definite change in nuclei of the Yoshida sarcoma cell.
[b] C.E.: Cytological effect upon the Yoshida sarcoma cell.
[c] P.L.: Prolongation of life of the tumor-rat treated by the compound.
[d] +: Positive effect, −: Negative effect.

the N-oxide derivatives of this series, contrary to our expectation, fell far below that of the parent substance, and consequently their mode of reaction in the animal body should be somewhat different from that of the N-oxides of ordinary nitrogen mustards.

CONCLUSION

With our aim to obtain useful derivatives of nitrogen mustards, N-bis-(β-chloroethyl)glycine, -alanine, -taurine, and their derivatives were prepared, and results of the screening test employing ascites (Yoshida) sarcoma of rats were described.

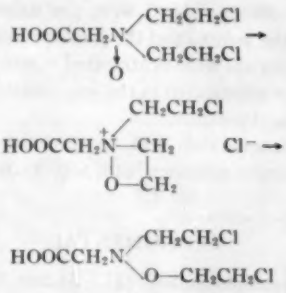
The first two amino acid derivatives were synthesized by acid hydrolysis of N-bis(β-chloroethyl)-alkylcyanide, which was obtained by the simultaneous condensation of bis-(β-chloroethyl)-amine with hydroxyalkyl sulfonate and sodium cyanide.

In physiological liquids, such acid derivatives of nitrogen mustard should exist mainly as an amphoteric ion, in which the reactivity of the chlorine atom is controlled by the formation of an ammonium ion. Nevertheless, the comparatively easy formation of immonium ion of the ethylene ring was observed as an intermediate. It might reasonably be assumed that such alkylating agents having amphoteric properties should react with a substrate in a different manner according to the *p*H of the tissue applied.

It was of interest to find that N-bis(β-chloroethyl)-alanine had no vesicant action and was eighteen times less toxic on rats than HN2, while the minimum effective dose against ascite sarcoma cells (Yoshida sarcoma) was of the same value as HN2. When the free carboxyl group of the compounds was esterified or transformed to its amide their toxicity became nearly as high as HN2.

The N-oxide derivatives of N-bis(β-chloroethyl)-glycine, -alanine, and their esters and amides were also prepared and submitted to the screening test. None of these N-oxides was found to be improved in toxicity nor in efficacy.

These N-oxides undergo the following transformation in a neutral aqueous solution at room temperature.



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Antispasmodics. II. Synthesis of Some Beta-Dialkylaminopropionanilides*

By FRANK E. DI GANGI†

Some thirty compounds were prepared wherein β-chloropropionyl chloride was substituted with various amines in the beta position as well as in the acyl portion of the molecule. The amines used as substituents were benzylamine, cyclohexylamine, morpholine, piperidine, diethylamine, di-*n*-propylamine, di-*n*-butylamine, di-*n*-amylamine, and di-*n*-heptylamine. The substituents for the acyl chloride were aniline, *p*-nitroaniline, benzylamine, cyclohexylamine, morpholine, and piperidine. Many of these were prepared as the free bases as well as the hydrochloride salts. The compounds prepared have not yet been screened for pharmacological activity.

SINCE THE PREVIOUS PUBLICATION (1) on the phenyl esters of β-dialkylaminopropionic acid these compounds were tested for antispasmodic activity on isolated guinea-pig ileum contracted with methacholine iodide. It was found that the phenyl and *p*-acetylaminophenyl compounds possessed a very low order of activity. During this time a related series of compounds, the amides, were synthesized so that their activity might also be tested and compared. Although

* Received October 7, 1954, from the University of Minnesota, College of Pharmacy, Minneapolis.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
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the esters, which are isomeric with procaine, were known to be lacking in local anesthetic properties. Gaind and Vohra (2) prepared some diethyl and piperidino derivatives of acetyl and propionyl-amino anilides and found the *o*-amino-*β*-diethyl-aminopropionanilides to possess activity equivalent to cocaine but the onset time, however, is longer.

In the series of the *β*-dialkylaminopropion-amides reported, the amines which were used as substituents in the *beta* position were benzyl-amine, hexylamine, morpholine, piperidine, di-ethylamine, di-*n*-propylamine, di-*n*-butylamine, di-*n*-amylamine, and di-*n*-heptylamine.

The amides prepared were the unsubstituted anilides, the *p*-nitro and the *p*-amino anilides. A few compounds were synthesized where the same amine was substituted in the *beta* position as well as in the acyl position.

These compounds will be screened for both antispasmodic and anesthetic activity in the near future.

EXPERIMENTAL

β-Chloropropionanilide (3).—Aniline (0.1 mole) was dissolved in 85 cc. glacial acetic acid and to this solution was added *β*-chloropropionyl chloride (0.11 mole) with stirring. A solution of sodium acetate (33 Gm. in 138 cc. water) was then added with stirring and the amide precipitated out immediately. The shaking was continued for ten minutes before the precipitate was filtered by suction, washed with water and allowed to air-dry. The yields on several runs averaged 90% of the theoretical. The m. p. was 115–117°. On recrystallization from alcohol it melted at 119° as reported (4). It is important that the solution of aniline in glacial acetic acid be chilled and the acid chloride added slowly in small portions to achieve the high yields reported here.

β-Chloro-*p*-nitropropionanilide.—The procedure was the same as that used in preparing the unsubstituted anilide. The volume of glacial acetic acid was increased to dissolve all the *p*-nitroaniline. The yield obtained was 66% of the theoretical. Melting point found was 171° as was reported (2).

β-Alkylaminopropionanilides.—*β*-Chloropropion-anilide (0.1 mole) was dissolved in 150 cc. benzene. To this warm solution was added a solution of the amine (0.25 mole) in 50 cc. benzene and the mixture was then refluxed for a period of time (three to six hours) necessary to obtain the theoretical amount of the by-product amine hydrochloride. After filtering off the amine hydrochloride, the filtrate was dried over anhydrous sodium sulfate, the benzene and the excess amine were removed by vacuum distillation. To the ether solution of the residue was added a solution of hydrogen chloride in ether and the product precipitated immediately from the solution as the hydrochloride. The product was filtered off and then air-dried. The cyclohexylamine, benzylamine, morpholine, and piperidine reacted well and in high yields. The products were crystallized from absolute alcohol. The same reaction when attempted with diethyl-amine resulted only in an oil even after the ethereal solution of hydrogen chloride was added. No attempts were made with the other dialkylamines on hand.

β-Alkylamino-*p*-nitropropionanilides.—The same procedure was used as for the *β*-dialkylaminopropionanilides except that the *β*-chloro-*p*-nitropropionanilide was suspended in benzene. In certain cases, the free bases of the *β*-alkylamino-*p*-nitropropionanilides were obtained as solids such as when cyclohexylamine, morpholine, piperidine, and diethylamine were used as substituents. The free base would appear after removal of the by-product, the benzene, and the excess amine, when ether was added to the residue.

β-Alkylamino-*p*-aminopropionanilides.—The *p*-nitropropionanilides were reduced to the *p*-amino compounds in the following general procedure: One gram of a 5% Pd-C catalyst, 50 cc. absolute alcohol to each 2.0 Gm. of the hydrochloride or the free base of the *p*-nitro compound. The solution was reduced with hydrogen under an initial pressure of 40 pounds. The reductions were rapid but were allowed to proceed for two hours. To the filtrate was added some ethereal hydrogen chloride. A precipitate formed immediately which was then recovered and purified in the manner indicated in Table II.

N,N′-Disubstituted β-Aminopropionamides.—*β*-Chloropropionyl chloride (0.11 mole) was dissolved in 200 cc. benzene and the amine (0.44 mole) was dissolved in 50 cc. benzene. The two solutions

TABLE I.—*β*-SUBSTITUTED *p*-NITROPROPIONANILIDES

	Substituent	Reaction Time, Hrs.	% Yield	Melting Point, °C. HCl	Melting Point, °C. Base	Chloride Analysis Calcd.	Chloride Analysis Found
1.	Benzylamine	15	80	225–226	54–55	10.56	10.57
2.	Cyclohexylamine	1	78	249–250	82–83	10.82	10.67
3.	Morpholine	4	98	255–256	95–96	11.27	11.14
4.	Piperidine	3	100	236–237	97–98[a]	11.30	11.10
5.	Diethylamine	4	75	189–190	95–96[b]	11.75	11.86
6.	Di-*n*-propylamine	2	68	197–198	147–148	10.75	10.66
7.	Di-*n*-butylamine	4	90	102–103	49–50	9.91	9.78
8.	Di-*n*-amylamine	3	86	135–136	Oil	9.19	9.19
9.	Di-*n*-heptylamine	3	50	119–120	Oil	8.02	8.09

Compounds 1–4 were recrystallized from alcohol; 5, 6 from absolute alcohol-ethyl acetate mixtures; 7 from absolute alcohol and Skelly B; 9 from a mixture of ether and absolute alcohol.

[a] Melting point reported, 100° (2).

[b] Melting point reported, 96° (2).

TABLE II.—β-SUBSTITUTED p-AMINOPROPIONANILIDES

	Substituent	% Yield	Melting Point, °C. ·HCl	Melting Point, °C. Base	Chloride Analysis Calcd.	Chloride Analysis Found
1.	Benzylamine	80	252 dec.	...	20.70	21.12
2.	Cyclohexylamine	85	150–151 [b]	96–97	11.90	11.79
3.	Morpholine	88	224–226	...	22.0	21.90
4.	Piperidine	93	243–244	96–97 [a]	22.15	22.18
5.	Diethylamine	64	180–181 [b]	...	13.05	13.07
6.	Di-*n*-propylamine	40	Over 250 decompn.	...	21.1	21.33
7.	Di-*n*-butylamine	82	214.5	...	19.46	19.38
			158–159 [b]	...	10.82	10.70
8.	Di-*n*-amylamine	76	129–130	...	18.0	18.10
9.	Di-*n*-heptylamine	62	162–163	...	15.80	15.60

[a] Melting point reported, 96° (2).
[b] These compounds are the monohydrochlorides of the reduced compounds.

TABLE III.—SUBSTITUTED β-AMINOPROPIONANILIDES

Substituent	Reaction Time, Hr.	% Yield	Melting Point, °C. ·HCl	Melting Point, °C. Base	Chloride Analysis Calcd.	Chloride Analysis Found
Benzylamine	6	100	256–257	Oil	12.12	12.04
Cyclohexylamine	4	100	213–214	83–84	12.50	12.60
Morpholine	4	86	229–230	44–45	13.10	13.16
Piperidine	3	100	214–215	57–58	13.20	13.35

TABLE IV.—N,N′-DISUBSTITUTED β-AMINOPROPIONAMIDES [a]

Substituent	Reaction Time, Hr.	% Yield	Melting Point, °C. ·HCl	Melting Point, °C. Base	Chloride Analysis Calcd.	Chloride Analysis Found
Benzylamine	..	..	...	...	...	...
Cyclohexylamine	3	75	248–249	42–43	12.30	12.36
Morpholine	24	96	205–206	93–94	13.40	13.23
Piperidine	18	50	202–203	...	13.60	13.80

[a] The disubstituted propionamides were recrystallized from alcohol-ethyl acetate mixture.

were mixed and refluxed a sufficient time to allow for the theoretical amount of the by-product to be recovered. The reaction mixture was allowed to stand overnight and was then filtered to remove all the by-product. The filtrate would sometimes yield the product as the free base at this point if it was a solid when the benzene was removed by vacuum distillation. Ethereal hydrogen chloride was then added to an ether solution of the residue obtained after the benzene was removed and the product precipitated out as the hydrochloride and recrystallized from a mixture of absolute alcohol and ethyl acetate. The reaction using benzylamine yielded a substance which proved later to be the benzylamide of β-chloropropionic acid with a m. p. of 93–94°. This same compound was prepared by an alternate method and showed no depression when mixed melts were taken. This melting point agrees with that of Hibicon® (5) which is the trade name for benzochlorpropamide.

When diethylamine was used in this reaction only an oil was obtained after treatment with the solution of hydrogen chloride in ether.

Preparation of the Free Bases.—In certain cases the amides as the free base solidified and/or were thrown out of solution after ether was added to the residue remaining when the reaction solvent and the excess amine were removed by vacuum distillation. In other instances the free base of the amide was prepared by alkalinizing an aqueous solution of the hydrochloride salt with dilute ammonia. The solution usually clouded and fine crystals formed or an oil would result.

SUMMARY

In this work some thirty compounds were prepared as the hydrochlorides and all but two are reported for the first time. The bases of these compounds were prepared in many cases. In the dialkylamine series, diethylamine did not yield a solid product with β-chloropropionyl chloride nor with β-chloropropionanilide, only oils resulted. The reaction between benzylamine and β-chloropropionyl chloride yielded only the intermediary product benzochlorpropamide which is Hibicon® instead of the desired disubstituted propionamide.

These compounds reported will be screened in the near future for pharmacological activity.

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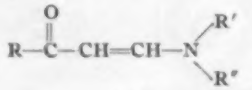
The Structure of Substances Which Affect the Autonomic Nervous System. II. Aminovinyl Ketones*

By GEORGE P. HAGER and JACOB S. HANKER†

In a continuing investigation of the effect of interaction of amino groups with adjacently oriented groups on pharmacodynamic properties, a series of aminovinyl ketones has been prepared. The preparation of these compounds was greatly facilitated by the finding that esters of hydroxyvinyl ketones with various acids react as vinylogous acid anhydrides on aminolysis.

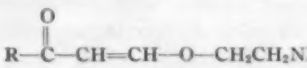
IN A FURTHER STUDY of the hypothesis that the molecular grouping on the carbon atom adjacent to one which bears an amine function is an important determinant of pharmacodynamic activity because of interaction of the adjacently oriented grouping or the carbon atom which bears it, with the amine function (1), it was of interest to prepare compounds in which an adjacently oriented carbonyl group is conjugated through a vinyl group with an amine function (I). Compounds of this type are vinylogous



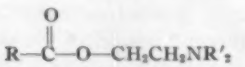
(I)

with amides as regards the interaction of the carbonyl group with the amino group, and may be considered aminovinyl ketones or vinylogous amides.

The original purpose of this investigation was the preparation of vinylogs (II) of cholinergic, spasmolytic, and locally anesthetic esters (III).



(II)

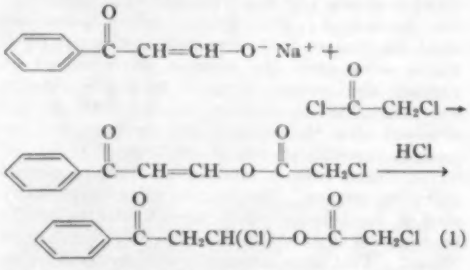


(III)

If the interaction of the acyloxy grouping with the amino group in compounds of type II is a determinant of pharmacodynamic activity, then any enhancement of the interaction of the carbonyl group with the "ether" oxygen should alter the interaction of the acyloxy grouping with the amine function and incur a change in pharmacodynamic activity.

For the preparation of compounds of type II, the reaction of N-(chloroalkyl)-dialkylamines with the sodium salts of hydroxyvinyl ketones (enols of β-ketoaldehydes or vinylogs of carboxylic acids), obtained by formylation of acetophenone or other ketones in the presence of sodium ethoxide, was found to be unsuccessful. If the esters were produced by this reaction, they could not be recovered from the reaction mixture. A crystalline hydrochloride was obtained from an ether-soluble, acid-soluble fraction, which had a m. p. (135–136°) which indicated that it might be the hydrochloride of 2-diethylaminoethanol, reported m. p. 134–135° (2).

The sodium salts of the hydroxyvinyl ketones were found to react quite vigorously with chloroacetyl chloride to produce the chloroacetates of the hydroxyvinyl ketones which form unstable hydrochlorides presumably as shown in Eq. 1.



The hydrochloride was first isolated from the reaction mixture and was later prepared from the unsaturated ester by treatment with a solution of hydrogen chloride in dioxane.

In attempts to produce aminoacetates and other esters by aminolysis of the chloroacetates and other halogen-substituted esters, aminovinyl ketones were formed. This was observed when the same product, 1-diethylamino-3-phenylpropen-3-one hydrochloride, was obtained by treating benzoylvinyl chloroacetate, benzoylvinyl β-chloropropionate, benzoylvinyl α-bromopropionate, and benzoylvinyl acetate with diethylamine in ether, followed by dry hydrogen

* Received August 27, 1954, from the School of Pharmacy, University of Maryland, Baltimore.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
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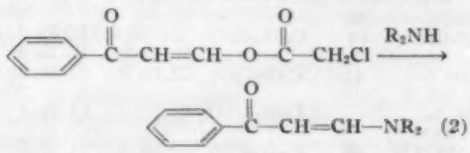
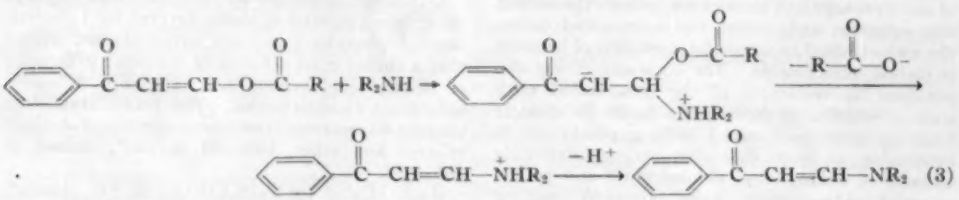
chloride. It became quite obvious that the esters were acting as vinylogs of acid anhydrides (Eq. 2). In subsequent studies, the unsubstituted acetates were found to be equally useful for the preparation of the aminovinyl ketones. In this



(2)

reaction, the acyloxy group is substituted by a nucleophilic attack of the amine on the carbon which is beta to the carbonyl since this carbon is rendered strongly electrophilic in the same way that the carbon *beta* to a carbonyl in the "pseudo-acidic" acceptor component of a Michael condensation is rendered electrophilic (3). The system is stabilized by elimination of the acyloxy group which is probably considerably less nucleophilic than the carbonanion that would be formed as intermediate in the rearrangement of the zwitterion to produce an adduct of the type normally formed in a Michael condensation (Eq. 3).



(3)

On the basis of the above-postulated mechanism it might be expected that the rate of substitution of the chloroacetate would be greater than that of the acetate, since the chloroacetate ion is less strongly nucleophilic than the acetate ion. In work now in progress, esters of still stronger acids are being studied as intermediates for the preparation of aminovinyl ketones and related compounds.

Aminovinyl ketones of the type included in this report have been made by reaction of the sodium salt of the hydroxyvinyl ketone directly with the amine hydrochloride, a reaction resembling the preparation of an amide from an acid and an amine (4). A second method which has been used for the preparation of aminovinyl ketones involved the addition of ammonia or amine to ethynyl ketones, intermediates which were somewhat less readily available than those employed in the work here reported (5).

Since the esters of the hydroxyvinyl ketones appear to react as vinylogous acid anhydrides, it should be possible to substitute the acyloxy group using nucleophilic substances other than ammonia or amine. Substitution of this group using an amino alcohol or its salt would produce the vinylogs of chlolinergic, spasmolytic, and locally anesthetic esters (III) which could not be prepared by the method previously tried. The preparation of such compounds is now being carried out.

EXPERIMENTAL

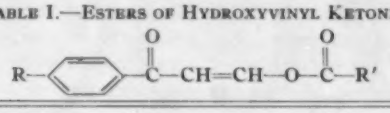
All melting points are uncorrected and were determined with a Fisher-Johns melting-point apparatus.

Preparation of Sodium Salts of Aryl-β-Hydroxyvinyl Ketones.—In 200 ml. of absolute ethanol was dissolved 23 Gm. (1 gram-atom) of sodium. When the solution was complete, the solvent was distilled off under reduced pressure and the residue suspended in 300 ml. of dry ether. To the stirred suspension of sodium ethoxide a mixture of 1 mole of acetophenone or other ketone and 74 Gm. (1 mole) of ethyl formate was slowly added. After the sodium salt of the hydroxyvinyl ketone had separated, the mixture was chilled and filtered. Yields of 80–90% were generally obtained, and the product could be recrystallized from a mixture of absolute ethanol and ether. In the following, the product was employed without recrystallization.

Esters of Aryl-β-Hydroxyvinyl Ketones.—To a chilled suspension of the sodium salt of the aryl-β-hydroxyvinyl ketone in 50 ml. of dioxane was added with stirring over a period of five minutes an equimolecular portion of an acid chloride (or 2 mole equivalents of acetic anhydride in preparation of acetates). When the exothermic reaction was complete, the mixture was treated with 500 ml. of cold water and shaken thoroughly to dissolve sodium chloride and any water-soluble unreacted starting material. The mixture was then chilled and stirred until the insoluble oil which separated, crystallized The yellow crystalline ester of the hydroxyvinyl ketone was filtered and was generally of sufficient purity for subsequent reactions. It could be recrystallized from methanol or petroleum ether with a minimum of heating on a steam bath. The esters prepared by this general method are listed in Table I.

If 2 mole equivalents of chloroacetyl chloride were used in the above procedure, the oil produced when the reaction mixture was treated with water, did not crystallize. The oil was separated and carefully dissolved in cold methanol. On further cooling, crystals separated which could be recrystallized from cold methanol. The product obtained in this way was most unstable, losing hydrogen chloride spontaneously at room temperature. On the basis

TABLE I.—ESTERS OF HYDROXYVINYL KETONES



Compound	R	R′	m. p.	Yield, %	Formula	Analysis Calcd.	Analysis Found
Benzoylvinyl acetate	H	—CH_3	69–70	90[a]			
p-Anisoylvinyl acetate	CH_3O	—CH_3	97–98	84	$C_{12}H_{12}O_4$	C, 65.4; H, 5.5	C, 65.3; H, 5.5
Benzoylvinyl chloroacetate	H	—CH_2Cl	100.5–101.5	41	$C_{11}H_9ClO_3$	Cl, 15.8	Cl, 15.6
Benzoylvinyl chloroacetate hydrochloride	H	—CH_2Cl	88.5–89.0	...	$C_{11}H_{10}Cl_2O_3$	Cl, 27.1	Cl, 26.7
p-Anisoylvinyl chloroacetate	CH_3O	—CH_2Cl	125–126	69	$C_{12}H_{12}ClO_4$	Cl, 14.0	Cl, 13.7
p-Anisoylvinyl chloroacetate hydrochloride	CH_3O	—CH_2Cl	88.5–89.0	25	$C_{12}H_{13}Cl_2O_4$	Cl, 25.8	Cl, 24.9
Benzoylvinyl β-chloropropionate	H	—CH_2CH_2Cl	58.0–58.5	69	$C_{12}H_{11}ClO_3$	Cl, 14.9	Cl, 14.4
p-Anisoylvinyl β-chloropropionate	CH_3O	—CH_2CH_2Cl	79–80	51	$C_{13}H_{13}ClO_4$	Cl, 13.2	Cl, 12.8
Benzoylvinyl α-bromopropionate	H	—$CHBrCH_3$	Unstable liquid				
p-Anisoylvinyl α-bromopropionate	CH_3O	—$CHBrCH_3$	Unstable liquid				

[a] Reported m. p. 70–72° (6).

of its chlorine analysis, it appeared to be an adduct of the ester of the hydroxyvinyl ketone and hydrogen chloride, and such products are listed in Table I as hydrochlorides. Whereas the unsaturated esters of the hydroxyvinyl ketones are yellow, the adduct was colorless; and, unlike the unsaturated esters, the adduct failed to decolorize a solution of bromine in carbon tetrachloride. The same adduct was also obtained by treatment of the unsaturated ester with a solution of hydrogen chloride in dioxane (melting point and mixed melting point). It is interesting to note that the hydrogen chloride adducts of benzoylvinyl chloroacetate and *p*-anisoylvinyl chloroacetate have identical melting points (88.5–89.0°). However the melting point of a mixture of the two was lower (79–84°), and benzoic acid was obtained by alkaline permanganate oxidation of one of them, while anisic acid was obtained from the other.

1-Diethylamino-3-phenylpropen-3-one Hydrochloride.—A solution of 19 Gm. (0.1 mole) of benzoylvinyl acetate in 75 ml. of anhydrous ether was chilled in an ice bath and treated with 14.6 Gm. (0.2 mole) of diethylamine, added in portions over a period of five minutes with continued cooling and stirring. The mixture was refluxed on a steam bath for thirty minutes, chilled, filtered if necessary, and treated with dry hydrogen chloride. The precipitated yellow solid was recrystallized from a mixture of absolute ethanol and ether, producing 18 Gm. (61%) of yellow crystalline solid, m. p. 128–131°.

Anal.—Calcd. for $C_{13}H_{18}ClNO$: N, 5.8. Found: N, 5.6.

The same product was obtained by use of the chloroacetate or the 2-chloropropionate in place of the acetate, in yields of 75 and 67%, respectively. The rather wide melting range of this compound and of the related compounds described below probably resulted from stereoisomerism involving the ethylenic bond as discussed by Bowden, *et al.* (5).

The free base, 1-diethylamino-3-phenylpropen-3-one, was prepared by treatment of an aqueous solution of the hydrochloride with ammonia solution, and recrystallization of the precipitate from petroleum ether. Pale yellow needles, m. p. 53°. Reported m. p. 53° (5).

1-Dimethylamino-3-phenylpropen-3-one Hydrochloride.—Prepared as above directed for 1-diethylamino-3-phenylpropen-3-one hydrochloride, except that a chilled ether solution of 9.5 Gm. (0.05 mole) of benzoylvinyl acetate was treated with excess anhydrous dimethylamine. The yellow crystalline product was recrystallized from a mixture of absolute ethanol and ether, softened at 135°, melted at 165–169°.

Anal.—Calcd. for $C_{11}H_{14}ClNO$: N, 6.6. Found: N, 6.6.

1-Diethylamino-3-(4-methoxyphenyl)-propen-3-one Hydrochloride.—Prepared by the procedure described for 1-diethylamino-3-phenylpropen-3-one hydrochloride from *p*-anisoylvinyl acetate (53%), *p*-anisoylvinyl chloroacetate (59%), and *p*-anisoylvinyl 2-chloropropionate (61%). The yellow crystalline product melted at 135–139°.

Anal.—Calcd. for $C_{14}H_{20}ClNO_2$: N, 5.2. Found: N, 5.1.

1-(1-Piperidyl)-3-(4-methoxyphenyl)-propen-3-one Hydrochloride.—A solution of 4.42 Gm. (0.02 mole) of *p*-anisoylvinyl acetate in 20 ml. of dioxane was treated with 3.40 Gm. (0.04 mole) of piperidine at room temperature. When the exothermic reaction was complete, ligroin was added to the reaction mixture to precipitate an oil. The oil was dissolved in a solution of dry hydrogen chloride in absolute ethanol and the hydrochloride precipitated by addition of ether. The product was crystallized from a mixture of absolute ethanol and ether; 1.5 Gm. (27%), m. p. 191–195°.

Anal.—Calcd. for $C_{15}H_{22}ClNO_2$: N, 5.0. Found: N, 4.7.

1-(4-Morpholinyl)-3-(4-methoxyphenyl)-propen-3-one Hydrochloride.—A solution of 4.42 Gm. (0.02 mole) of *p*-anisoylvinyl acetate in 50 ml. of methanol was treated while cooling in an ice bath and stirring, with 3.48 Gm. (0.04 mole) of morpholine. When

the exothermic reaction was complete, water was added to precipitate an oil which was washed with ligroin. The oil dissolved in ethanol and crystals of the free base were obtained by chilling the solution. The free base was converted to the hydrochloride in the usual way, and the latter was recrystallized from a mixture of absolute ethanol and ether. Yellow crystalline solid, 2.2 Gm. (39%), m. p. 112–115°.

Anal.—Calcd. for $C_{18}H_{18}ClNO_3$: N, 4.9. Found: N, 5.2.

1 - (4-Methoxyphenylamino) - 3 - (4 - methoxyphenyl)-propen-3-one.—A chilled solution of 22.1 Gm. (0.1 mole) of *p*-anisoylvinyl acetate in 100 ml. of ethanol was treated with 24.6 Gm. (0.2 mole) of *p*-anisidine while cooling and stirring. The product crystallized in the reaction mixture and was recrystallized from ethanol. Yellow crystals, 14.8 Gm. (52%), m. p. 184–185°.

Anal.—Calcd. for $C_{17}H_{17}NO_4$: N, 4.9. Found: N, 5.1.

A bright orange hydrochloride prepared from the free base underwent discoloration rapidly on standing.

1-Phenylamino-3-phenylpropen-3-one.—Prepared by the procedure described for the preparation of 1-(4-methoxyphenylamino)-3-(4-methoxyphenyl)-propen-3-one, using benzoylvinyl acetate and aniline. Yellow crystals, m. p. 139–140°.

Anal.—Calcd. for $C_{15}H_{13}NO$: N, 6.3. Found: N, 6.2.

1-(4-Ethoxyphenylamino)-3-phenylpropen-3-one.—Prepared by the procedure described for the preparation of 1-(4-methoxyphenylamino)-3-(4-methoxyphenyl)-propen-3-one, using benzoylvinyl acetate and *p*-phenetidine. Yellow crystals, m. p. 150–151°.

Anal.—Calcd. for $C_{17}H_{17}NO_3$: N, 5.2. Found: N, 5.2.

1-(2-Pyridylamino)-3-(4-methoxyphenyl)-propen-3-one Dihydrochloride.—A mixture of 22.1 Gm. (0.1 mole) of *p*-anisoylvinyl acetate and 9.4 Gm. (0.1 mole) of 2-aminopyridine in 100 ml. of methanol was heated to boiling on a steam bath. The mixture was cooled and treated with water to precipitate the crude amino ketone. The solid was dissolved in a solution of anhydrous hydrogen chloride in absolute ethanol and the yellow crystalline dihydrochloride was precipitated by addition of ether. The product was recrystallized from a mixture of absolute ethanol and ether, 4.3 Gm. (13%), m. p. 135–139°.

Anal.—Calcd. for $C_{15}H_{16}Cl_2N_2O_2$: N, 8.6. Found: N, 9.1. In all likelihood a certain amount of the monohydrochloride was present in the product.

N,N′-Bis(benzoylvinyl)-ethylenediamine.—To a solution of 9.5 Gm. (0.05 mole) of benzoylvinyl acetate in 60 ml. of methanol was added with cooling and stirring 4.5 Gm. (0.075 mole) of ethylenediamine. When the exothermic reaction was complete, the mixture was chilled thoroughly, and a yellow crystalline product obtained and recrystallized from methanol, 6.2 Gm. (39%), m. p. 143–144°, reported m. p. 142° (5).

N,N′ - Bis(*p* - anisoylvinyl) - ethylenediamine.—Prepared from *p*-anisoylvinyl acetate by the procedure described for N,N′-bis(benzoylvinyl)-ethylenediamine. The prouuct was recrystallized from ethyl acetate. Yellow crystals, 5.6 Gm. (59%), m. p. 218–219°.

Anal.—Calcd. for $C_{22}H_{24}N_2O_4$: N, 7.4. Found: N, 7.6.

SUMMARY

1. In a study of methods for preparation of vinylogs of various cholinergic, spasmolytic, and locally anesthetic esters; a convenient procedure for the preparation of aminovinyl ketones was developed.

2. The preparation of benzoyl- and *p*-anisoylvinyl esters and their behavior as vinylogous anhydrides on aminolysis are described.

3. A series of aminovinyl ketones was prepared for pharmacologic study in a continuing investigation of the effect of interaction of amino groups with adjacently oriented groups on pharmacodynamic properties.

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Determination of Salicylic Acid and Two Metabolites in Plasma and Urine Using Fluorimetry for Directly Measuring Salicyluric Acid*

By EDWARD B. TRUITT, JR., ANN M. MORGAN, and J. MAXWELL LITTLE

An improved method has been developed for the measurement of salicylic acid and salicyluric acid in blood plasma and for these two substances and salicyl glycuronates in urine. Older methods measured salicyluric acid by the different extraction efficiencies of two solvents and the weaker color formation of salicyluric acid with ferric nitrate. This method measures salicyluric acid more specifically by means of its marked fluorescence at *p*H 10. A low concentration of a fluorescent substance, probably salicyluric acid, which has not been reported previously was found in dog plasma following large doses of sodium salicylate. The rate of hydrolysis of salicyluric acid by concentrated hydrochloric acid, an important factor in the calculation of the salicyl glycuronate fraction in this and former methods, has been re-examined and a higher value than previously reported has been found. An explanation of this difference is suggested. A synthetic sample of the glycuronate of salicylic acid coupled through the phenolic hydroxyl group, which has not been isolated from urine in pure form, has been tested for reactivity in the method.

SINCE THE CHEMICAL CHARACTERIZATION of the urinary metabolites of salicyclic acid (SAL) by Kapp and Coburn (1) and the development of a simple method for the measurement of SAL in blood plasma by Brodie, Udenfriend, and Coburn (2) considerable interest has been manifested in the study of SAL and its metabolites in blood and urine. Smith, *et al.* (3), developed a method for the determination of SAL and its derivatives in urine. Their method for salicyluric acid (SUA) was indirect depending upon differences in the extraction efficiency for SUA of carbon tetrachloride (CCl_4) compared to ethylene dichloride (EDC) and differences in the color formation of SAL and SUA with ferric nitrate. Their method for the salicyl glycuronates (SG) depended upon the complete hydrolysis of this metabolite by concentrated hydrochloric acid in three hours and determination as SAL with calculation of the per cent of SUA hydrolyzed in that time. This method has been used with minor modifications by Hoffman and Nobe (4) and by Cronheim, *et al.* (5). A similar method has also been developed by Dalgaard-Mikkelsen (6).

In experimenting with a fluorimetric method for blood salicylate, developed by Saltzman (7), it was found that SUA has a markedly greater fluorescence than SAL, especially at *p*H 10, where a maximum in the molar fluorescence ratio occurred. This suggested the possibility of a more direct method of measuring SUA in urine and possibly detecting the metabolite in plasma. Brodie, *et al.* (2), were unable to detect SUA in plasma by the differential extraction method.

The method reported here utilizes the poorer extraction of SUA by CCl_4 and its weaker color formation as observed by Smith, *et al.* (3), to provide a method with a 50:1 specificity for SAL compared to SUA on a molar ratio basis. By utilizing the greater fluorescence of SUA at *p*H 10 after extraction with EDC, which extracts SAL and SUA equally well, a method with a 40:1 ratio favorable to SUA has been developed. This permits the direct determination of SUA with a small correction for large proportions of SAL. The phenolic hydroxyl-coupled glycuronate of salicylic acid, which is the only derivative excreted in the dog and is the principal form of salicyl glycuronate in human urine (8), is neither fluorescent nor reactive with ferric nitrate until after hydrolysis.

The SG fraction is determined in this method by simple subtraction of the calculated amount of SUA hydrolyzed by the concentrated hydrochloric acid plus the free SAL from the total SAL measured after three hours acid hydrolysis. The use of simultaneous equations to calculate SUA and SG as in previous methods is not required. A different figure from those previously reported has been obtained for the calculation of SUA hydrolysis in three hours and reasons for this difference are suggested.

EXPERIMENTAL

Reagents.—(*a*) Ethylene dichloride, technical grade, purified by successive washings with NaOH, HCl, and H_2O. (*b*) Ethylene dichloride with 1.5% isoamyl alcohol. Isoamyl alcohol, reagent grade, is added to ethylene dichloride purified as in (*a*) to 1.5%. (*c*) Carbon tetrachloride, reagent

* Received September 18, 1954, from the Laboratories of the A. H. Robins Co., Inc., and the Department of Physiology and Pharmacology, The Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

grade, b. p. 75–77°. (*d*) Hydrochloric acid, 6 *N*. (*e*) Hydrochloric acid, concentrated—37%. (*f*) Ferric nitrate, 1% in 0.7 *N* nitric acid. (*g*) Sodium chloride, reagent grade. (*h*) Salicylic acid stock solution, 1.0%. 1.16 Gm. of sodium salicylate is dissolved in 100 ml. of water. Each milliliter of this solution will contain the equivalent of 10 mg. of salicylic acid. Appropriate dilutions of this solution are employed for preparation of standard reference curves. Store in the refrigerator. (*i*) Salicyluric acid stock solution, 1.412%: 1.412 Gm. of salicyluric acid is dissolved in 100 ml. of water. This solution will also contain the equivalent of 10 mg. of salicylic acid per milliliter. Appropriate dilutions of this solution are employed for preparation of standard reference curves. Store in the refrigerator. (*j*) Salicyluric acid standard solution, 0.1% (for standardizing fluorimeter). 100 mg. of salicyluric acid is dissolved in 100 ml. H_2O. Store in the refrigerator. Dilutions of 1:100 of this solution with *p*H 10 buffer are made daily. (*k*) *p*H 10 buffer solution (approximately *p*H 10 at 18°.)—Kolthoff and Vlesschhower's alkali soda-borax buffer.[1] 75.4 ml. Solution A (Na_2CO_3,0.53%) plus 24.6 ml. Solution B ($Na_2B_4O_7 \cdot 10H_2O$,0.191%).

Colorimetric Determination of Free SAL in Plasma and Urine.—The method which has been employed for the colorimetric determination of free SAL was suggested by the method described by Brodie, *et al.* (2), as modified by Smith, *et al.* (3). By using CCl_4, which extracts SUA very poorly compared to its efficiency in extracting SAL, a specificity of extraction for SAL is obtained. This advantage combined with the weaker color formation of SUA with ferric nitrate gives a total color formation by SUA after extraction with CCl_4 of only 2% that of an equimolar amount of SAL. For the proportions of SUA relative to SAL in all plasmas and in dog urine this amount of interference may be disregarded. In human urine a correction may be necessary in the colorimetric free SAL value because of the presence of a larger amount of SUA.

Procedure.—Place 1.0 ml. of plasma or diluted urine (1:10), 1.0 ml. of water, and 0.5 ml. of 6 *N* HCl in a 50-ml. glass-stoppered centrifuge tube containing 30.0 ml. of CCl_4. Shake for five minutes at a 45° angle,[2] and then spin for five minutes to separate. Remove by aspiration the supernatant aqueous layer and discard. Transfer exactly 20.0 ml. of the CCl_4 layer to another 50-ml. glass-stoppered centrifuge tube containing 10.0 ml. of water and 0.25 ml. of the $Fe(NO_3)_3$ solution. Shake for five minutes at a 45° angle[2] and then centrifuge for five minutes. Transfer at least 6.0 ml. of the aqueous phase to a dry colorimeter tube and read in an Evelyn or other suitable colorimeter using a filter with maximal transmission at 540 mμ. Known concentrations of sodium salicylate (NaSAL) were prepared in pooled human plasma ranging from 1 to 50 mg. % (as SAL). These were carried through this procedure to provide a standard reference curve. This is necessary because the extraction efficiency of CCl_4 for SAL is less from plasma than from water. Similarly prepared concentrations of SAL in urine ranging from 10 to 500 mg. % were diluted 1:10 with water and then analyzed by this procedure to give a standard reference curve for urine. SAL is extracted equally well from water or dilute urine by CCl_4. Blank samples of plasma and urine have been tested and repeatedly found to be devoid of chromogenic material. With human urine, though not with dog urine, a correction of the colorimetric reading may be necessary for a high proportion of SUA. If required, this curve should be made by carrying through the same procedure 1.0-ml. samples of SUA in urine ranging from 100 mg. to 1000 mg. %. Representative standard reference curves obtained by this method are shown in Fig. 1.

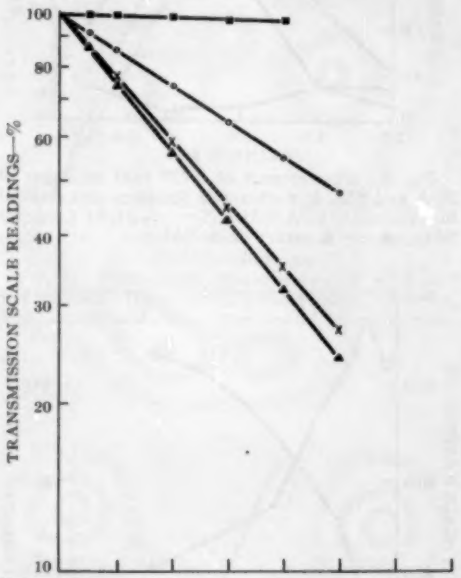

Fig. 1.—Colorimetric determinations of SUA and SAL extracted from urine and pooled human plasma. X——X SAL, plasma; ▲——▲ SAL, urine; ○——○ SAL, hydrolysis-urine; ■——■ SUA, urine.

Fluorimetric Determination of SUA in Plasma and Urine.—For this determination we have used a Beckman spectrophotometer adapted for fluorescence using the same filters as for the U. S. P. thiamine hydrochloride assay (maximum transmission of primary 370 mμ, secondary 460 mμ). Both SAL and SUA exhibit a fluorescence in alkaline solutions. However, it was found by testing equimolar solutions of SAL and SUA in various buffers of *p*H 7.0 and above that a maximum in the fluorescence ratio of SUA divided by SAL occurred about *p*H 10. This relationship of fluorescence to *p*H is shown in Fig. 2. At *p*H 10 the relationships between fluorescence and concentration of equimolar amounts of SUA and SAL are shown in Fig. 3. Although at higher concentrations the fluorescence from SUA is less in proportion to concentration, in the portion of

[1] Clark, W. M., "Hydrogen Ions," 3rd ed., Williams & Wilkins Co., Baltimore, Md., 1928.

[2] Five minutes shaking at this angle has been found sufficient for maximal extraction in this laboratory. However, the minimum time for maximal extraction should be determined for the apparatus employed.

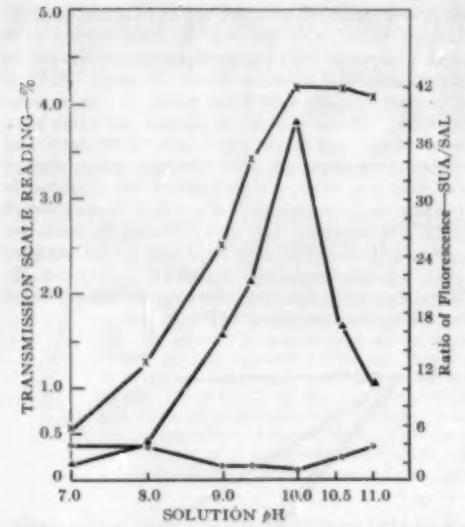

Fig. 2.—Fluorescence of 0.723 mM solutions of SUA and SAL in various *p*H solutions and ratio of fluorescence of SUA/SAL. X——X SUA; ○——○ SAL; ▲——▲ ratio—SUA/SAL.

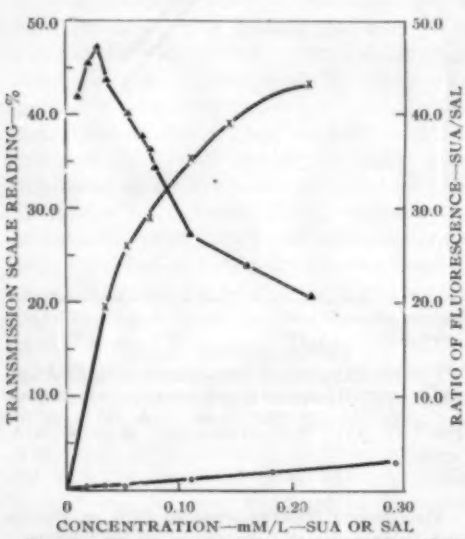

Fig 3.—Fluorescence of SUA and SAL in *p*H 10 buffer solution and fluorescent ratio of equimolar solutions of SUA/SAL. X——X SUA; ○——○ SAL; ▲——▲ Ratio—SUA/SAL.

the curve where the relation between fluorescence and concentration is close to linear the ratio of fluorescence of SUA to SAL is about 40:1 on a molar basis and greater on a weight basis. The ratios of fluorescence for equimolar solutions of SUA to SAL are also plotted in Fig. 3. For these reasons *p*H 10 was selected for development of a method highly specific for SUA. EDC was selected for extraction since Smith, *et al.* (3), have shown that both SAL and SUA are efficiently and equally extracted by this solvent. No solvent could be found which extracted SUA preferentially. Both the addition of 1.5% isoamyl alcohol and an excess of NaCl increased the extraction efficiency of this solvent for SUA and SAL. The addition of NaCl was used for extractions from water and dilute urine but not from plasma.

Procedure.—Place either 2.0 ml. of plasma and 0.5 ml. of 6 *N* HCl or 1.0 ml. of diluted urine (1:10), 1.0 ml. of water, and 0.5 ml. of 6 *N* HCl in a 50-ml. glass-stoppered centrifuge tube with 30.0 ml. of EDC containing 1.5% isoamyl alcohol. Add 1.5 Gm. of NaCl for extractions from urine. Shake for five minutes on a shaking machine at a 45° angle. Spin for five minutes at moderate speed. Remove by aspiration the upper aqueous layer and discard. Transfer exactly 20.0 ml. of the EDC phase to a glass-stoppered centrifuge tube containing 5.0 ml. of the *p*H 10 buffer. Shake for five minutes at a 45° angle and then centrifuge for five minutes. Remove with a pipet 2.0 ml. of the *p*H 10 buffer phase and add it to 4.0 ml. of the *p*H 10 buffer in a test tube (add it to only 2.0 ml. in the case of plasma extractions to increase fluorescence). Mix well and transfer 4.0 ml. to a cuvette for reading in a fluorimeter. A cuvette filled with *p*H 10 buffer is used for the zero setting and a solution of SUA containing 0.01 mg./ml. (0.0071 mg./ml. as SAL) in *p*H 10 buffer is used to adjust the sensitivity of the instrument so that this solution gives a transmission scale reading of 25.0%.

Using this procedure, standard reference curves for the determination of SUA and SAL extracted from plasma and urine were prepared. Concentrations of SUA ranging from 0.1 to 1.2 mg. % as SAL and concentrations of NaSAL ranging from 1.0 to 60.0 mg. % as SAL were added to pooled human plasma and carried through the procedure. These curves for plasma are shown in Fig. 4. Concentrations of SUA ranging from 2.0 to 180 mg. % as SAL and concentrations of NaSAL ranging from 100 to 3000 mg. % as SAL were prepared in urine, diluted 1:10 with water, and carried through the procedure. The curves for urine are shown in Fig. 5.

Although the fluorescence due to SAL is slight, the usual ratio of SAL/SUA in plasma and in most urine samples is so great that the apparent (or total) SUA value obtained by fluorescence must be corrected for the SAL. This is accomplished as follows: The known free SAL concentration, colorimetrically obtained, is converted to a fluorescence equivalent by reference to the appropriate standard curve relating extracted SAL concentration to fluorescence. The concentration of extracted SUA (as SAL) equivalent to this fluorescence is then obtained from the appropriate extracted SUA standard curve. The true SUA concentration (as SAL) is then obtained by subtraction of the SUA equivalence of the SAL present.

Plasma and urine blank samples must be carried through the extraction procedure and subtracted from the total fluorescence in the same manner as the correction for SAL fluorescence. Plasma blank fluorescence is often not present and has been consistently lower for a 2.0-ml. sample than has that for a 1.0-ml. urine sample. Urine blank fluorescence varies between individuals but has been relatively constant in a given subject. Urine blank fluorescence in one dog during 28 experiments gave a mean transmission reading of 0.33% with a standard

deviation of 0.13%. This mean is equivalent to 2.0 mg. % of SUA.

Determination of Salicyl Glycuronates in Urine.—In this method, as in that of Smith, *et al.* (3), the determination of the SG depends upon complete hydrolysis by concentrated hydrochloric acid of the glycuronic ester linkage in less than three hours, and a calculation of the amount of salicyluric acid which will be hydrolyzed in three hours.

A synthetic sample of the glycuronate[1] of salicylic acid coupled through the phenolic hydroxyl group was analyzed and found neither to be fluorescent nor to react with ferric nitrate until after a ten-minute hydrolysis with concentrated HCl. The color formed with ferric nitrate after hydrolysis was equivalent to the calculated amount of SAL present. These data are shown in Table 1.

TABLE I.—FLUORIMETRIC AND COLORIMETRIC ANALYSIS OF O-(β-D-GLUCURONOSIDO)-SALICYLIC ACID (AHR-81) BEFORE AND AFTER HYDROLYSIS FOR TEN MINUTES WITH CONCENTRATED HCl

		Concentration as SAL—mg. %			
		Before Hydrolysis		After Hydrolysis	
Sample No.	SAL as AHR-81 Added	Colorimetric	Fluorimetric	Colorimetric	Recovery, %
1	50	0	0	47.5	95.0
2	50	0	0	47.5	95.0
3	50	0	0	48.1	96.2
4	Blank	0	0	0	..

A large discrepancy exists in the reports by various investigators of the per cent of SUA hydrolysis after three hours exposure to concentrated hydrochloric acid in a boiling water bath. Smith, *et al.* (3), reported that 33% of the SUA was hydrolyzed in three hours with this treatment. Hoffman and Nobe (4) realized that the extraction efficiency of EDC for SAL and SUA from aqueous solutions containing concentrated HCl is less than that from aqueous solutions of 6 *N* HCl. They calculated the per cent hydrolysis of SUA using a reference curve prepared by heating known concentrations of SAL in concentrated HCl for three hours in a boiling water bath with subsequent extraction by EDC. Their value for SUA hydrolysis in three hours was 46.7%. Cronheim, *et al.* (5), reported a value of 79% for the three-hour hydrolysis value of SUA. They gave no details as to how they obtained this figure but did suggest that the weaker color produced upon extraction from concentrated HCl is due to the carrying over of HCl into the final step of the procedure. Dalgaard-Mikkelsen (6) reported complete hydrolysis of SUA in his method but this was after hydrolysis with both acid and alkali.

Smith, *et al.* (3), state that the hydrolysis reaction of SUA is a monomolecular one since the log of per cent SUA unhydrolyzed is a straight line function of time. Although this may be true, Smith and his co-workers related the per cent increment of the theoretically possible total increment in absorbance during hydrolysis of SUA directly to the per

[1] The synthetic sample of the SAL-glycuronate coupled through the phenolic hydroxyl group of SAL (O-(β-D-glucuronosido)-salicylic acid) was prepared by Dr. Carl D. Lunsford, A. H. Robins Co., Inc. Richmond, Va. Details of synthesis and composition will be the subject of a separate communication.

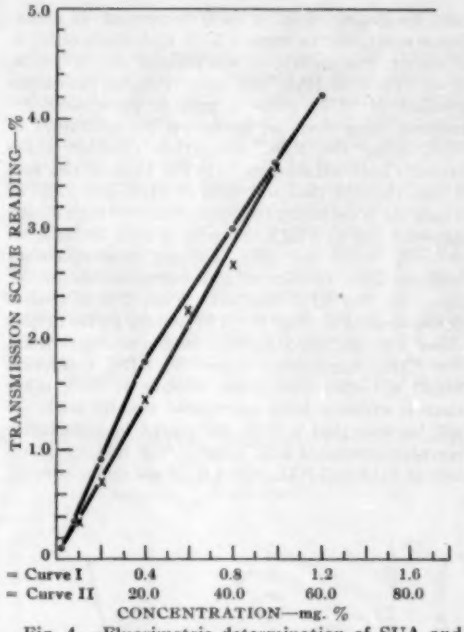

Fig. 4.—Fluorimetric determination of SUA and SAL extracted from pooled human plasma. X——X SUA, expressed as SAL equivalents; O——O SAL.

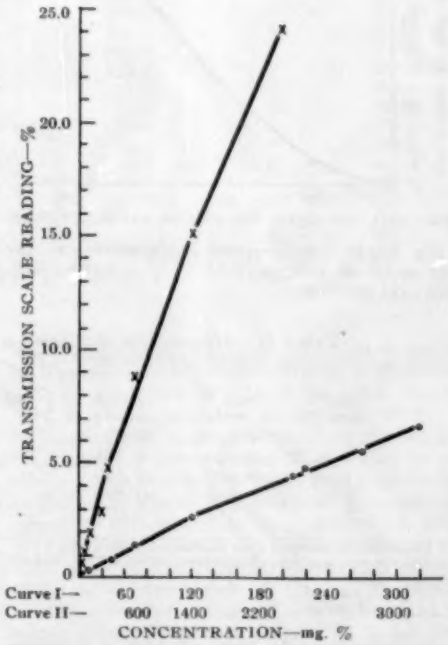

Fig. 5.—Fluorimetric determination of SUA and SAL extracted from urine. X——X SUA, expressed as SAL equivalents; O——O SAL.

cent hydrolysis, i. e., a 34% increment in absorbance was taken to mean a 34% hydrolysis of SUA. However, it is known, as was pointed out by Smith, *et al.* (3), that SAL, the color-reactive hydrolysis product of SUA, gives a greater absorbance increment than does an equimolar concentration of SUA using the EDC extraction method which extracts both substances. On the basis of this fact it was thought that mixtures of SUA and SAL, in which the total molar concentration was maintained constant but in which the ratio of SAL to SUA increased, would not give a direct proportionality between SAL increment and increment of absorbance by the EDC method. That this is true is demonstrated in Fig. 6. The values given in this curve were obtained directly on the mixtures without EDC extraction. However, EDC extraction would not alter the values relative to each other since it extracts both substances equally well. It will be seen that a 34% increment in absorbance is representative of a 61% increase of SAL in a mixture of SUA and SAL, when the total molar concentration is constant. Thus a 34% increase in absorbance during hydrolysis of SUA would represent a 61% hydrolysis, instead of the 34% hydrolysis reported by Smith, *et al.* We have tested repeatedly two samples of SUA[4] for degree of hydrolysis by measuring the increment in color upon extraction with CCl_4. In this procedure (extraction with CCl_4) there is practically no color at the start of the hydrolysis because SUA is so poorly extracted, and the increment of color with time is a measure of the moles of SAL produced by hydrolysis. We have also calculated the hydrolysis in three hours by measuring the SUA disappearance by fluorimetric analysis. Using the former method we have obtained a figure of 61.53% ± a S. D. of 6.23%. The hydrolysis value obtained by a reinterpretation of the data of Smith, *et al.*, as given above, agrees closely with the value obtained by us using two different methods.

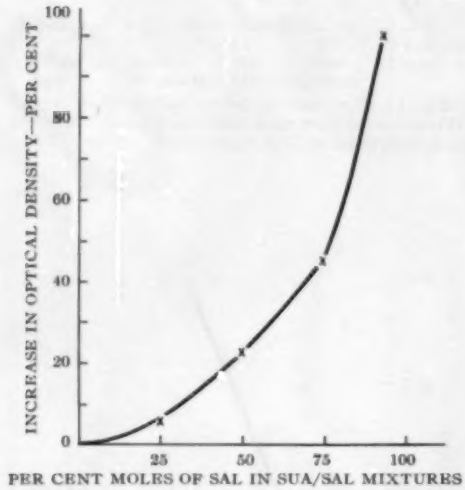

Fig. 6.—Per cent increase in absorbance *vs.* per cent moles of salicylic acid in a salicyluric/salicylic acid mixture.

In Table II are shown the results of a typical experiment measuring the hydrolysis rate of SUA over a period of three hours by the calculation both from moles of SAL appearing and moles of SUA disappearing.

Using this hydrolysis figure of 62% instead of the others reported in the literature the following method has been used to measure the glycuronate fraction in urine.

Procedure.—A 0.5 ml.-sample of diluted urine (1:10 or occasionally 1:20) and 1.0 ml. of concentrated HCl are placed in a 15-ml. graduated conical centrifuge tube with a loosely fitting ground-glass stopper. The sample is hydrolyzed for three hours by immersing the lower part of the tube in a boiling water bath. The sample is removed from the bath, cooled rapidly, and diluted to 2.5 ml. with water. The sample is then transferred to a 50-ml. glass-stoppered centrifuge tube and carried through the same procedure as for the colorimetric determination of free SAL. A reference curve must be made by submitting 0.5-ml. urine samples of known SAL concentration ranging from 50 to 500 mg. % to this procedure. This is important since there is a less efficient extraction when concentrated HCl is used instead of 6 *N* HCl. The concentration of SAL obtained after hydrolysis for three hours (ST) represents the sum of three fractions. These are (*a*) the free SAL, (*b*) 62% of the SUA, and (*c*) the SG, all three expressed as mg. % of SAL. Therefore the SG fraction is obtained as mg. % of SAL by subtracting from the total SAL after hydrolysis (ST) 62% of the SUA plus all of the free SAL. This is represented by the equation:

$$SG = ST - (\text{free SAL} + 0.62\ SUA)$$

TABLE II.—HYDROLYSIS OF A SAMPLE CONTAINING 0.1805 MM SUA BY CONCENTRATED HCl

Time, Hrs.	SAL,[a] mM	SUA,[b] mM	Total SAL and SUA, mM	% Hydrolyzed—mM SAL / 0.1805 mM	% Hydrolyzed—mM SAL / Total mM
0	0	0.179	0.179	0	0
½	0.029	0.164	0.193	16.1	15.0
1	0.049	0.146	0.195	27.1	25.1
2	0.083	0.094	0.177	46.0	46.8
3	0.114	0.078	0.192	63.1	59.5

[a] Colorimetric analysis after extraction with CCl_4.
[b] Fluorimetric analysis after extraction with EDC and correction for SAL.

[4] We are indebted to Dr. Norman H. Leake, S. K. Massengill Co., for one sample of salicyluric acid. Results with this sample were identical to those of a sample synthesized for us by Dr. Robert S. Murphey, A. H. Robins Co., Inc., Richmond, Va.

RESULTS

An illustration of the application of this method to the determination of SAL and SUA in aqueous mixtures having various ratios of the two compounds is shown in Table III. The same ratios of SAL:SUA were determined also in urine and the results shown in the same table. Also in the table are the results obtained upon analysis of three mixtures of SAL and SUA added to plasma in the approximate ratio found in dogs receiving large intravenous doses of sodium salicylate.

The recovery of SAL or SUA seems to vary similarly to other procedures with a corresponding number of manipulations except that accuracy declines somewhat when either compound is greatly in excess. Recovery from urine is slightly more variable than from water.

DISCUSSION

Because of the wide discrepancy in the reported percentages of SUA hydrolyzed in three hours by concentrated HCl (3, 4, 5), no attempt was made to compare recoveries by this method with results obtained using the method of differential extraction of SUA by CCl_4 and EDC. The factors which we believe contribute to these differences were pointed out in the discussion of the method of measuring the SG fraction of the salicyl metabolites in urine.

Brodie, *et al.* (2), were unable to detect any difference between CCl_4 and EDC extraction of plasma and thus concluded that there was a negligible amount of SUA in the plasma of human subjects. By our method of analysis a fluorescent substance appears in the plasma of dogs following the administration of large doses of sodium salicylate which

TABLE III.—EXTRACTION OF MOLAR MIXTURES OF SAL-SUA FROM WATER, URINE, AND POOLED PLASMA

Molar Ratio: SAL/SUA	SAL—mg. % Added	SAL—mg. % Recovered	Recovery, %	SUA—mg. % Added	SUA—mg. % Recovered	Recovery, %
			Water			
1:20	2.0	2.1	105.0	56.5	...	...
1:10	2.0	2.05	100.5	28.2	29.5	104.5
1:4	5.0	4.98	99.8	28.2	27.0	95.8
1:2	10.0	9.98	99.8	28.2	27.8	97.5
1:1	20.0	19.8	98.0	28.2	26.9	95.5
2:1	20.0	19.8	99.0	14.1	13.5	95.8
4:1	20.0	20.0	100.0	7.06	6.9	97.8
10:1	20.0	20.2	102.0	2.82	3.0	106.2
20:1	40.0	41.0	102.5	2.82	2.97	105.2
40:1	40.0	41.0	102.5	1.41	1.57	111.1
		Av. =	100.9		Av. =	101.0
			Urine			
1:20	20.0	21.0	105.0	565.0	...	...
1:10	20.0	19.0	95.0	282.0	280.0	99.4
1:4	50.0	47.0	94.0	282.0	277.5	98.5
1:2	100.0	91.0	91.0	282.0	273.0	96.8
1:1	200.0	186.0	93.0	282.0	276.0	97.0
2:1	200.0	199.0	99.6	141.0	120.0	85.1
4:1	200.0	192.0	96.1	70.6	61.0	86.5
10:1	200.0	190.0	95.0	28.2	30.0	106.5
20:1	400.0	400.0	100.0	28.2	28.0	99.4
40:1	400.0	400.5	101.2	14.1	15.0	106.4
		Av. =	97.0		Av. =	97.3
			Plasma			
20:1	20.0	20.0	100.0	1.41	1.41	100.0
20:1	10.0	10.0	100.0	0.706	0.75	106.1
20:1	5.0	4.8	96.0	0.353	0.45	127.5

In Table IV are shown the results of an application of this method to the analysis of plasma and urine samples from a dog which received a single intravenous dose of 100 mg./Kg. of sodium salicylate.

In this experiment no effort was made to regulate the urine *p*H in either an acid or alkaline range. This would alter the relative proportions of SAL, SUA, and SG excreted (3). It will be seen that urine *p*H shifted toward a lower value after the third hour. Salicyluric acid excretion was not detectable in this dog's urine when the *p*H was above 7.0. Measurable quantities of a fluorescent substance in plasma, which is not accountable chemically as SUA or SG, appear in this dog to parallel the occurrence of SUA in the urine. This substance in plasma appears to be SUA.

is not accountable as SAL or SG. Correlation has been noted between this fluorescence in the plasma and the appearance of SUA in the urine. We believe that this substance is, perhaps, SUA. In agreement with Dalgaard-Mikkelsen (6) we find that a greater proportion of SUA occurs in acid urines. This may be seen in the data from the dog in Table IV where SUA appeared in measurable quantities only when the urine acidity fell below *p*H 7.0.

In none of the previously described methods (3–6) has a pure sample of a salicyl glycuronate been available for testing. However, based on the statements of Kapp and Coburn (1) it has been assumed that the glycuronates do not react with ferric nitrate until after acid hydrolysis. In this study we have confirmed this nonreactivity until

TABLE IV.—SALICYLATE METABOLITES IN DOG PLASMA AND URINE FOLLOWING INTRAVENOUS INJECTION OF 100 MG./KG. OF SODIUM SALICYLATE

Time after Injection, Hrs.	1	3	6	9	12	24
Urine—*p*H[a]	7.5	7.7	7.0	6.4	6.0	6.5
Plasma concentrations—mg. %[b]						
Salicylate	31.8	27.0	17.5	13.0	9.0	4.0
Salicylurate	0.0	0.0	0.0	0.14	0.10	0.12
Urine excretion rates—mg./hr.[b]						
Salicylate	24.5	67.5	88.0	4.9	2.1	2.2
Salicylurate	0.0	0.0	0.0	2.1	4.0	16.0
Salicyl glycuronate	24.5	52.5	83.7	19.9	27.0	97.0

[a] Measured with a Leeds-Northrup glass-electrode *p*H meter.
[b] Measured as salicylic acid.

after hydrolysis for the glycuronate of salicylic acid coupled through the phenolic hydroxyl group by testing a synthetic sample. This glycuronate also does not contribute to the fluorescence of EDC extracts before hydrolysis. An attempt is being made to obtain synthesis of a glycuronate coupled to salicylic acid through the carboxyl group.

SUMMARY

1. A quantitative method has been developed which measures salicylic acid (SAL) and salicyluric acid (SUA) in plasma and SAL, SUA, and the salicyl glycuronates (SG) in urine.

2. The measurement of SUA is by the direct measurement of the fluorescence of that compound at *p*H 10 with a small correction for the slight fluorescence of SAL at that *p*H. This is in contrast to other methods (3–6) in which SUA is measured indirectly by the difference in extraction efficiency of two solvents for this substance and the weaker color formation of this compound with ferric nitrate.

3. Using this method a fluorescent substance is detectable in plasma which appears to be SUA.

4. The rate of hydrolysis of SUA, which is an important factor in the calculation of the SG fraction in urine, has been retested and a different value found. Reasons for the difference between this figure and those previously reported are suggested.

5. A synthetic sample of the glycuronate of salicylic acid coupled through the phenolic hydroxyl group has been tested to check the lack of reactivity of this metabolite with ferric nitrate until after hydrolysis and also for lack of fluorescence before hydrolysis.

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N-(2-Hydroxy-5-Chlorobenzylidine)-Anilines and N-(2-Hydroxy-5-Chlorobenzyl)-Anilines*

By DAVID B. REISNER and PAUL M. BORICK

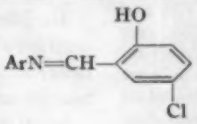
Fourteen N-(2-hydroxy-5-chlorobenzylidine)-anilines and eleven N-(2-hydroxy-5-chlorobenzyl)-anilines have been prepared and tested for *in vitro* antibacterial and antifungal activities. Both classes of compounds were found to possess a high degree of activity.

THE RECENT PUBLICATION of Farrow, *et al.*, (1) on the antifungal activity of certain azomethines, including N-(2-hydroxy-5-chlorobenzylidine)-4-carboxyaniline, has prompted us to report here our work along similar lines.

As part of a chemotherapy program in our laboratories, series of N-(2-hydroxy-5-chlorobenzylidine)-anilines and N-(2-hydroxy-5-chlorobenzyl)-anilines were synthesized. The Schiff bases were conveniently prepared, usually in excellent yield and in a high state of purity, by mixing together alcoholic solutions containing equimolecular quantities of 5-chlorosalicylaldehyde and the corresponding aromatic amine (Table I). Catalytic reduction of the Schiff bases

* Received December 3, 1954, from the Research Laboratories of Wallace & Tiernan Incorporated.

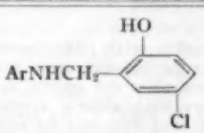
TABLE I.—N-(2-HYDROXY-5-CHLOROBENZYLIDINE)-ANILINES



Ar	m. p., °C.[a]	Yield, %	Nitrogen, % Calcd.	Found	Antibacterial and Antifungal Activities, p. p. m. *T. intermedia*	*M. audouini*	*C. albicans*	*E. coli*	*S. aureus*
Phenyl	111–112	94	6.05	5.95	30	10	100	100	30
p-Tolyl	154–155	96	5.70	5.69	10	10	>30[b]	>30	>30
2,4-Xylyl	116–117	94	5.39	5.25	10	10	>30	>30	>30
p-Chlorophenyl	149.5–150.5	97	5.26	5.25	10	10	>30	>30	>30
m-Chlorophenyl	127–128	97	5.26	5.31	10	10	30	100	100
2,4-Dichlorophenyl	133–134	93	4.66	4.66	10	10	>30	>30	>30
p-Carboxyphenyl	300–302	99	5.08	5.02	10	5	>30	>30	>30
o-Carboxyphenyl	199–200	77	5.08	5.09	10	10	100	100	100
p-Nitrophenyl	192–194	87	10.13	10.20	30	10	100	100	100
p-Dimethylaminophenyl	169–170	89	10.20	10.30	>30	>30	>30	>30	>30
p-Hydroxyphenyl	242–243	85	5.66	5.67	10	10	100	>100	>100
p-Methylmercaptophenyl	143–144	90	5.04	5.10	30	100	>100	>100	>100
1-Naphthyl	101–102	85	4.97	4.97	10	5	30	>30	>30
4-Methyl-2-pyridyl	155–156	83	11.36	11.52	30	10	100	100	300

[a] Melting points are uncorrected.
[b] The symbol > indicates that the compound precipitated out of the medium at the concentration reported.

TABLE II.—N-(2-HYDROXY-5-CHLOROBENZYL)-ANILINES



Ar	m. p., °C.	Yield, %	Nitrogen, % Calcd.	Found	Antibacterial and Antifungal Activities, p. p. m. *T. intermedia*	*M. audouini*	*C. albicans*	*E. coli*	*S. aureus*
Phenyl	115–116	70	5.99	5.95	30	10	100[c]	>1,000	10
p-Tolyl	114–114.5	71	5.65	5.60	10	10	100	> 100	10
2,4-Xylyl	87–88	70	5.35	5.15	10	10	>100	> 100	13
p-Chlorophenyl	86.5–87.5	86	5.22	5.08	3	3	30	> 100	3
m-Chlorophenyl	97–98	79	5.22	5.20	3	3	30	100	3
2,4-Dichlorophenyl	73.5–74.5	65	4.63	4.56	3	3	10	> 100	0
p-Carboxyphenyl	203–204	78	5.04	5.02	>100	>100	>100	> 100	100
o-Carboxyphenyl	138–139	64	5.04	4.80	100	30	>100	> 100	30
p-Hydroxyphenyl[a]	127–129	78	5.61	5.55	30	30	300	300	30
1-Naphthyl	110–111	88	4.94	4.85	3	3	30	> 100	3
4-Methyl-2-pyridyl·HCl[b]	221–223	41	9.83	9.96	30	100	>300	> 300	>300

[a] This compound was isolated after reduction by neutralizing the acetic acid with aqueous sodium bicarbonate.
[b] This compound was isolated after reduction by neutralizing the acetic acid with aqueous sodium bicarbonate, extracting with ether, and saturating the dried ethereal solution with anhydrous HCl.

over platinum oxide in acetic acid proceeded smoothly to yield the secondary amines (Table II).

EXPERIMENTAL

Intermediates.—The aromatic amines were commercial products and were used without further purification. 5-Chlorosalicylaldehyde was prepared according to Durrans (2) using two moles of salicylaldehyde and four moles of sulfuryl chloride. The yield of 5-chlorosalicylaldehyde, m. p. 101–102°, was 94%. 4-S-Methylmercaptoaniline was prepared by reducing *p*-nitrophenylmethylsulfide (3) according to Ferry, *et al.*, (4). The product was condensed with the aldehyde without further purification.

N-(2-Hydroxy-5-Chlorobenzylidine)-anilines.—To 1 mole of 5-chlorosalicylaldehyde in 600 ml. of warm absolute ethanol was added 1 mole of the aromatic amine in 200 ml. of warm absolute ethanol. The mixtures were chilled in an ice bath for one hour and filtered. The solids were removed, washed with 95% ethanol, and recrystallized from absolute ethanol. Yields and melting points are recorded in Table I.

N-(2-Hydroxy-5-chlorobenzyl)-anilines.—A mixture consisting of 0.2 mole of the N-(2-hydroxy-5-chlorobenzylidine)-anilines, 300 mg. of platinum oxide catalyst, and 250 ml. of acetic acid was shaken with hydrogen (initial pressure, 40 lb.) in a Parr hydrogenation apparatus at room temperature until reduction was complete (approx. 60 min.). After removal of catalyst, products were precipitated with water and recrystallized from absolute ethanol.

Yields and melting points are recorded in Table II.

Microbiological Tests. *E. coli* ATCC #26 and *Micrococcus pyogenes* var. *aureus* FDA #235 were grown on nutrient agar or tryptone-glucose agar for eighteen to twenty-four hours at 37°. Cells were harvested with sterile saline and 1 ml. of inoculum was added to 9 ml. of nutrient broth. Serial dilutions, in the order of 1, 3, 10, of the compound under test were added and the tubes were incubated for forty-eight hours at 37°. The highest dilution showing no growth was taken as the end point.

Trichophyton interdigitale #598 and *M. audouini* ATCC #10008 were grown on Sabouraud's agar at room temperature for seven to ten days and the activity of the compound was assayed as above in Sabouraud's broth. *Candida albicans*, a recent isolate, was treated exactly like the fungi except that it was studied at 37°.

The results of the microbiological tests are included in Tables I and II.

SUMMARY

1. The preparation and antibacterial and antifungal activities of N-(2-hydroxy-5-chlorobenzylidine)-anilines and N-(2-hydroxy-5-chlorobenzyl)-anilines are described.

2. Both types of compounds exhibit a high degree of activity.

3. The most active members of the series are the N-(2-hydroxy-5-chlorobenzyl)-chloroanilines and N-(2-hydroxy-5-chlorobenzyl)-1-naphthylamine.

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Tests for Rosin in Official Pharmaceuticals*

By LOUIS FISCHER and NATHAN A. HALL

Two tests for possible adulteration with rosin have been compared for cinnamon oil, juniper tar, linseed oil, tolu balsam, peruvian balsam, jalap resin, ipomea Resin, and Asafetida. The tests were the cupric acetate test, in which rosin was detected by treating an extract with cupric acetate, and the Lieberman-Storch-Morawski test, in which rosin was detected by treating the sample or an extract with acetic anhydride and sulfuric acid. The Lieberman-Storch-Morawski test was the more sensitive. When rosin adulterated samples of tolu balsam, jalap resin, ipomea resin, or asafetida are heated or aged, tests for rosin become weaker than for fresh samples. Thus, the adoption of a more sensitive test than the one now official for these substances appears to be indicated.

FOR A NUMBER OF YEARS the test for rosin as an adulterant of official resins, balsams, and oils has been a subject of controversy. Although a general method has been included in the U. S. P. (1), it has not been universally applicable. In order to establish a sound basis for selection and to correct certain inconsistencies, the problem of the test for rosin in pharmaceuticals was re-examined.

Rosin in soaps, paints, and varnishes has been extensively investigated, but only a few tests have survived for any period. Most of the work has been in the area of quantitative determination and modification of existing qualitative tests. These have been reviewed by Kopf, *et al.* (2), and by Sanderman (3). In the pharmaceutical field far less experimental work has been published (4–8) although record has been made of dissatisfaction with the rosin test (9).

* Received August 27, 1954, from the College of Pharmacy, University of Washington, Seattle 5.
Presented to the Scientific Section, A. PH. A., Boston meeting, August 1954.
The authors wish to thank H. L. Milner, Lloyd Gaines, Janis O. Runikis, and Ulla B. Forsstrom, students of the University of Washington College of Pharmacy, for their assistance with the experimental work.

In official compendia at present two basic tests are employed. The first, the cupric acetate test, involves the extraction of rosin from the sample with petroleum benzin, followed by treatment with cupric acetate to form green copper abietate (1). The second, the Lieberman-Storch-Morawski test, involves treatment of the sample with acetic anhydride followed by sulfuric acid to give an evanescent violet color (11).

EXPERIMENTAL

The Cupric Acetate Test.—Initially the test was tried on each official substance whose monograph included a test for rosin. The test was performed as outlined in the U. S. P. (1) on samples containing 1 and 5% rosin (Table I). A sample of known purity was used as a control in each instance.

An inconsistency in the concentration of cupric acetate used for the test in various procedures was noted, the test for cinnamon oil requiring a 1:1000 concentration, juniper tar 1:100, and the general test 1:200. Therefore the tests were run again with each of the three concentrations. In all instances a 1:100 solution of cupric acetate gave the most distinct and reliable results.

TABLE I.—CUPRIC ACETATE TEST FOR ROSIN

	Control	Rosin 1%	Rosin 5%
Cinnamon Oil U. S. P.	Colorless	Light green (S)[a]	Green (S)
Juniper Tar N. F.	Light brown	Light brown (U)[b]	Brown (U)
Linseed Oil N. F.	Green	Green (U)	Green (U)
Tolu Balsam U. S. P.	Colorless	Colorless (U)	Blue-green (S)
Peruvian Balsam U. S. P.	Colorless	Colorless (U)	Light blue-green (S)
Jalap Resin[c] N. F.	Colorless	Light green (S)	Green (S)
Ipomea Resin[c] N. F.	Colorless	Light green (S)	Green (S)
Asafetida N. F.	Colorless	Light green (S)	Green (S)

[a] Satisfactory—S. Unsatisfactory—U.
[b] When the test for Juniper Tar was carried out as directed in the N. F. IX monograph, in which the petroleum benzin layer is decanted into an equal volume of ether, the test was satisfactory.
[c] For Jalap and Ipomea Resins N. F. IX lists pink as the color indicating a positive test. The color is green not pink as observed formerly by Wirth (10).

The Lieberman-Storch-Morawski Test.—The test was run as outlined in the monograph for linseed oil (11) on all of the samples listed in Table I. Only for linseed oil was the test satisfactory. The test was then modified by extracting with petroleum benzin and running the color reaction on the petroleum benzin extract.

The procedure used was as follows: One gram of the substance was triturated in a small mortar with 10 ml. of petroleum benzin for one to two minutes and the extract filtered into a test tube. One milliliter of acetic anhydride was added to the filtrate followed by 1 drop (0.05 ml.) of concentrated sulfuric acid, and the test tube was shaken gently. In the presence of rosin an evanescent purple to violet color was obtained which faded to red and finally to brown. The red color was stable from ten to thirty minutes. A control in which no rosin was present was run concurrently. The results shown in Table II were obtained.

TABLE II.—MODIFIED LIEBERMAN-STORCH-MORAWSKI TEST FOR ROSIN

	Control	Rosin 1%	Rosin 5%
Cinnamon Oil U. S. P.	Purple	Purple (U)[a]	Purple (U)
Juniper Tar N. F.	Brown	Brown (U)	Brown (U)
Linseed Oil N. F.[b]	Brown	Violet (S)	Purple (S)
Tolu Balsam U. S. P.	Tan	Violet (S)	Purple (S)
Peruvian Balsam U. S. P.	Brown	Violet (S)	Purple (S)
Jalap Resin N. F.	Tan	Purple (S)	Purple (S)
Ipomea Resin N. F.	Brown	Purple (S)	Purple (S)
Asafetida N. F.	Tan	Violet (S)	Purple (S)

[a] Satisfactory—S. Unsatisfactory—U.
[b] No preliminary extraction with petroleum benzin.

The modified test was found by the authors to be sensitive to 0.1% rosin in the original sample. With the exception of linseed oil, the satisfactory tests were confirmed by the American Pharmaceutical Association Laboratory (12).

To determine whether another solvent would be more suitable than petroleum benzin, nineteen other solvents were tried. Only *n*-hexane, cyclohexane, ligroin (b. p. 60–90°), ligroin (b. p. 90–120°), and light liquid petrolatum were satisfactory. These solvents offered no advantages over petroleum benzin.

Several acids and dehydrating agents were examined as substitutes for sulfuric acid in the test. Of twelve substances investigated only perchloric acid and zinc chloride were satisfactory. Neither of these was as good as sulfuric acid. Variation of the strength of the sulfuric acid used in the test from 10% to concentrated (96%) was investigated. All strengths produced less stable colors than concentrated sulfuric acid.

When benzoyl chloride, glacial acetic acid, and acetyl chloride were tried as substitutes for acetic anhydride, none was found to be satisfactory.

Effect of Heat.—During the preparation of the samples with rosin adulteration, it was noted that if heat was used to incorporate rosin in the solid samples, variable results in the tests were obtained. Therefore a preliminary investigation was undertaken on the effect of heat on samples adulterated with rosin.

Both the Lieberman-Storch-Morawski and the cupric acetate tests were run on adulterated samples before and after heating. Since only one test was satisfactory for cinnamon oil, juniper tar, and linseed oil, only the official tests of their current monographs were used. The effect of heat upon samples containing 5% rosin gave the results shown in Table III.

In a number of cases both tests for rosin were lessened after heating; further experimentation revealed that if the adulterated samples were heated for only five minutes at 155°, the tests were noticeably weakened. One sample of tolu balsam containing 10% rosin was heated for seven days at 160–200°; at the end of this time, the modified Lieberman-Storch-Morawski test was markedly lessened but still positive.

Although the results shown are for samples containing 5% rosin, a similar weakening of the tests was shown when samples containing 10 and 20% rosin were heated. However, both tests effected satisfactory detection of rosin at these higher percentages.

Samples which were prepared without heat by trituration of the powdered substance and powdered rosin also exhibited a weaker test upon standing for thirty days. Thus it appears that heat may merely accelerate a reaction which would occur spontaneously.

SUMMARY AND CONCLUSIONS

1. No single test for rosin is completely suitable as a general test for use in all the official pharma-

TABLE III.—THE EFFECT OF HEAT IN SAMPLE PREPARATION ON ROSIN TESTS

	Unheated Control		Heated 10 Minutes at 160–180°		Heated for 1½ hours at 100°	
	Cupric Acetate	L-S-M[a]	Cupric Acetate	L-S-M	Cupric Acetate	L-S-M
Cinnamon Oil U. S. P.[b]	+++++	...	+++++	...	+++++	...
Juniper Tar N. F.	+++++	...	+++++	...	+++++	...
Linseed Oil N. F.	...	+++++	...	+++++	...	+++++
Tolu Balsam U. S. P.	+++++	+++++	+	+++	++++	++++
Peruvian Balsam U. S. P.	+++++	+++++	+++++	+++++	+++++	+++++
Jalap Resin N. F.	+++++	+++++	+	+++	+++++	+++++
Ipomea Resin N. F.	+++++	+++++	+	+++	++++	++++
Asafetida N. F.	+++++	+++++	+	+++	+++	++++

[a] L-S-M—Lieberman-Storch-Morawski test.
[b] The intensity of the reaction is indicated by plus signs.

ceutical substances. The cupric acetate test as outlined in the respective monographs is satisfactory for the detection of rosin in cinnamon oil and for juniper tar. For unheated, fresh samples of tolu balsam, peruvian balsam, asafetida, ipomea resin, and jalap resin the test produces satisfactory detection if the rosin adulteration is greater than 1%. However, this test may fail if the adulterated samples have been allowed to stand for some time or have been heated.

2. A uniform strength (1:100) of cupric acetate solution should be used.

3. The wording of the rosin test in the N. F. monographs for ipomea and jalap resin should be changed to read "the benzin layer does not assume a green color."

4. The Lieberman-Storch-Morawski test now official for linseed oil is satisfactory for this substance. As modified to include preliminary extraction with petroleum benzin, this test is suitable for all official pharmaceuticals except cinnamon oil and juniper tar. It is more sensitive than the cupric acetate test now official and is more reliable for aged or heated adulterated samples. The modified Lieberman-Storch-Morawski test is recommended as a substitute for the cupric acetate test for tolu balsam, peruvian balsam, asafetida, ipomea resin, and jalap resin.

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The Pharmacological Relationship of a Series of Pentenyl Substituted Barbituric Acid Derivatives*

By E. E. SWANSON, W. R. GIBSON, and W. J. DORAN

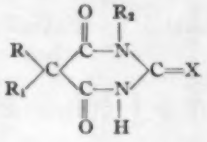
A series of pentenyl barbituric acid derivatives has been studied. In general, the amount required to produce anesthesia is independent of the duration of action. Furthermore, there is no obvious relationship between the pharmacological action and chemical structure of these compounds. Many of these derivatives are isomeric, but show considerable variation in action, dosage, duration of action and therapeutic index. One of these compounds is shorter acting than two well-known barbiturates and with a better therapeutic index.

IN A PREVIOUS COMMUNICATION (1) the obvious relationship between the pharmacological action and the chemical structure of certain barbituric acid derivatives was discussed. In the substitution of primary or secondary alkyl groups in one of the 5,5-positions, with an increasing number of C-atoms in the alkyl chain, both the anesthetic and the lethal dose grow relatively smaller, but when the alkyl radical was longer than 5 C-atoms, the amount required to produce anesthesia or death in rats again was increased.

* Received August 27, 1954, from the Divisions of Pharmacology and Organic Chemistry of the Lilly Research Laboratories, and the College of Pharmacy, Butler University, Indianapolis 6, Ind.
Presented to the Scientific Section of the A. PH. A., Boston meeting, August, 1954.
The authors are indebted to Miss Eva Sommermeyer and Mr. Wallace C. Wood for their invaluable assistance in this work, and to Dr. K. K. Chen for his many kind suggestions and criticisms.

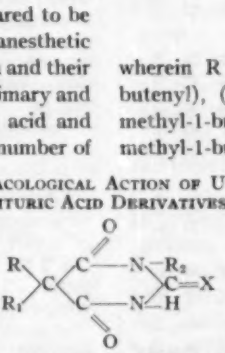
Furthermore, as the alkyl chain became longer, the therapeutic index, or ratio between the anesthetic and lethal doses, gradually appeared to be greater. In general, the duration of action showed a similar reaction; that is, it was shorter when the alkyl group became lengthened. In primary alkyl-substituted derivatives, the critical compound was one that possessed 6 C-atoms in one of the 5,5-positions, but with more than 6 C-atoms in either of the 5,5-positions the duration of action began to grow longer. The substitution of a methyl, ethyl, or allyl radical on one of the nitrogens (nitrogen alkyl-substituted barbituric acid derivatives) (2, 3); or the substitution of a methallyl (2-methyl-allyl) (4) or a crotyl (3-methyl-allyl) (5) on one of the 5,5-positions; or a sulfur atom (6) in place of the oxygen on the 2 C-atom, definitely reduced the duration of action. This shorter duration of action was independent of the amount of drug administered. It was observed that sheep's blood treated with a series of primary and secondary alkyl-substituted barbituric or thiobarbituric acid compounds, hemolyzed more rapidly as the number of C-atoms increased in the substituted alkyl radical (7). In several series of isomeric barbituric acid derivatives, there appeared to be a distinct relationship between the anesthetic and lethal dosage and duration of action and their chemical structure (8). In a series of primary and secondary alkyl-substituted barbituric acid and thiobarbituric acid compounds, as the number of C-atoms increased in the substituted alkyl chain the inhibition or sedative action increased on the isolated intestine of rabbits and frogs and on the isolated uterus of guinea pigs. The same degree of depressions was observed in the perfused frog's heart (9). More recently (10), in a series of spiro-barbituric and spiro-thiobarbituric acids, substitution on the alicyclic ring by single or multiple alkyl groups resulted in compounds possessing hypnotic and anesthetic properties. Intravenous administration to rats revealed structural relationships to activity. The spiro-thiobarbituric acids were long acting in rats, which indicated that these animals are not readily capable of metabolizing these substances. In rabbits and dogs, however, the spiro-thiobarbituric acids were shorter acting than their non-thio mates.

The present investigation is concerned with the study of a series of pentenyl alkyl barbituric acid derivatives, synthesized by one of us (W. J. D.) with the general formula:



wherein R may be a 2-pentenyl, (1-methyl-3-butenyl), (2-methyl-2-butenyl), 1-pentenyl, (1-methyl-1-butenyl), (1-ethyl-1-propenyl) or (3-methyl-1-butenyl); R_1 a methyl, ethyl, *n*-propyl,

TABLE I.—COMPARISON OF THE PHARMACOLOGICAL ACTION OF UNSATURATED ALKYL SUBSTITUTED BARBITURIC ACID DERIVATIVES



Administered Intravenously in Rats as 2% Solutions of the Sodium Salts

Compd. No.	R	R_1	R_2	X	No. of Rats	AD_{50} ± S.E. mg./Kg.	Min. Av. Duration of AD_{50}	LD_{50} ± S.E. mg./Kg.	Therapeutic Index
1	$C_2H_5CH{=}CHCH_2-$	C_2H_5	H	O	41	63.5 ± 5.7	61	148 ± 4.8	2.34
2	$C_2H_5CH{=}CHCH_2-$	C_2H_5	H	S	48	45.0 ± 2.7	75[a]	86 ± 7.1	1.91
3	$C_2H_5CH{=}CHCH_2-$	C_2H_5	CH_3	O	32	24.0 ± 2.0	15[b]	52 ± 2.3	2.16
4	$CH_2{=}CHCH_2CH(CH_3)-$	C_2H_5	H	O	32	73.0 ± 3.8	300[c]	86 ± 6.9	1.17
5	$C_2H_5CH{=}CHCH_2-$	*iso*-C_3H_7	H	O	96	50.0 ± 2.8	82	109 ± 3.1	2.18
6	$C_2H_5CH{=}CHCH_2-$	*iso*-C_3H_7	H	S	48	38.5 ± 2.5	96[c]	104 ± 4.9	2.70
7	$C_2H_5CH{=}CHCH_2-$	*iso*-C_3H_7	CH_3	O	48	44.5 ± 2.0	25[d]	73 ± 1.5	1.66
8	$CH_3CH{=}C(CH_3)CH_2-$	*iso*-C_3H_7	H	O	56	58.0 ± 1.6	102[c]	88 ± 4.6	1.50
9	$C_2H_5CH{=}CHCH_2-$	*n*-C_3H_7	H	O	48	103.0 ± 4.1	69	157 ± 4.4	1.50
10	$C_2H_5CH{=}CHCH_2-$	$CH_2{=}CHCH_2-$	H	O	30	88.0 ± 4.4	119	128 ± 5.0	1.45
11	$C_2H_5CH{=}CHCH_2-$	*sec*-C_4H_9	H	O	48	66.0 ± 2.9	67	100 ± 1.0	1.51
12	$CH_3CH_2CH_2CH{=}CH-$	*iso*-C_3H_7	H	O	48	60.0 ± 1.5	107[d]	107 ± 5.4	1.78
13	$CH_3CH_2CH{=}C(CH_3)-$	*iso*-C_3H_7	H	O	48	46.0 ± 1.6	97	86 ± 2.6	1.87
14	$CH_3CH{=}C(C_2H_5)-$	*iso*-C_3H_7	H	O	48	55.0 ± 1.5	115[d]	103 ± 5.7	1.87
15	$(CH_3)_2CHCH{=}CH-$	*iso*-C_3H_7	H	O	48	56.0 ± 2.2	125[d]	104 ± 2.5	1.85

[a] Postanesthetic tremors.
[b] Diaphragmatic spasms with tremors.
[c] Tremors.
[d] Preanesthetic tremors.

TABLE II.—COMPARATIVE PHARMACOLOGICAL ACTION OF 5-(2-PENTENYL)-5-ISOPROPYL BARBITURIC ACID (SODIUM SALT) WITH SECONAL® SODIUM AND AMYTAL® SODIUM

Species of Animal	Route of Administration	Compound No. 5 5-(2-Pentenyl)-5-isopropyl Barbituric Acid				Seconal® Sodium				Amytal® Sodium			
		$AD_{50} \pm$ S.E., mg./Kg.	Av. Duration Min.	$LD_{50} \pm$ S.E., mg./Kg.	LD AD	$AD_{50} \pm$ S.E., mg./Kg.	Av. Duration Min.	$LD_{50} \pm$ S.E., mg./Kg.	LD AD	$AD_{50} \pm$ S.E., mg./Kg.	Av. Duration Min	$LD_{50} \pm$ S.E., mg./Kg.	LD AD
Rats	I.V.	50 ± 2.8	82	109 ± 3.1	2.18	38.5 ± 1.6	129	65 ± 2.1	1.69	65 ± 3.7	200	128 ± 9.1	1.97
	Oral	240 ± 26.0	100	510 ± 65.0	2.13	74.0 ± 10.0	236	112 ± 5.0	1.51	220 ± 15.0	676	380 ± 20.0	1.73
Rabbits	I.V.	38 ± 2.6	142	64 ± 1.8	1.68	18.6 ± 0.6	161	37.5 ± 1.9	2.01	45 ± 1.5	200	70 ± 4.5	1.87
Dogs	I.V.	31 ± 1.5	212	78 ± 2.4	2.52	22.5 ± 3.0	413	48 ± 3.5	2.13	45 ± 2.4	920	75 ± 5.0	1.56
	Oral	71 ± 12.0	637	142 ± 3.3	2.00	32.5 ± 3.1	815	85 ± 7.5	2.30	66 ± 2.2	1340	112 ± 17.5	1.70

1-methylethyl, allyl or 1-methylpropyl; X a sulfur atom in place of the oxygen; and R_2 a methyl radical in place of the hydrogen on one of the nitrogens.

METHODS AND SPECIFIC COMPOUNDS

Albino rats weighing 80 to 120 Gm. (average 99 Gm.) were used. Solution of the sodium salts of the compounds were injected by vein. The anesthetic dose, duration of action, and lethal dose were determined by using eight or more rats for each dose level. The median anesthetic dose ($AD_{50} \pm$ S.E.) and the median lethal dose ($LD_{50} \pm$ S.E.) were read off from the logarithmic-probit paper devised by Miller and Tainter (11). During the tests the rats were kept in a warm room, the temperature of which ranged from 29.4 to 32.2°.

As shown in Table I, the isomeric pentenyl radicals on position R were either primary or secondary, with double bonds on the 1, 2, or 3 C-atoms. In compounds No. 1, 2, 3, 5, 6, 7, 8, 9, 10, and 11 the primary pentenyl group was the same, with a double bond on the 2 C-atom. In compound No. 4 the pentenyl radical was secondary, with a methyl group on the 1 C-atom and a double bond on the 3 C-atom. A primary pentenyl radical with a methyl group on the 2 C-atom and a double bond on the 2 C-atom, was in the R position as shown in compound No. 7. Compounds No. 12, 13, 14, and 15 were pentenyls with double bonds on the 1 C-atom, a primary pentenyl radical in compound No. 12, a secondary pentenyl in derivatives No. 13 and 14 with methyl and ethyl radicals on the 1 C-atom respectively and an isopentenyl in compound No. 15.

RESULTS

In general the amount (mg. per Kg.) required to produce anesthesia is independent of the duration of action. As shown in Table I with compound No. 2 the oxygen on the 2 C-atom is replaced by a sulfur. The anesthetic and lethal doses of this compound, and the therapeutic index are smaller. The duration of action, which after anesthesia is followed by postanesthetic tremors, is slightly longer than that of its oxygen analog compound No. 1. A methyl radical on the nitrogen lowers the anesthetic and lethal doses of compound No. 3 and shortens the duration of action to one-fourth (fifteen minutes) that of the parent compound No. 1. Compound No. 4 is isomeric with compound No. 1 but in place of the 2-pentenyl it has a secondary pentenyl (1-methyl-3-butenyl). Compound No. 4 is five times longer acting (three hundred minutes) than its parent compound No. 1. It requires a larger anesthetic dose and produces tremors or light convulsions. It is more toxic than compound No. 1 and has a very low therapeutic index. Compound No. 5, with a normal pentenyl (2-pentenyl) radical in position R and an isopropyl group in position R_1 has good anesthetic properties without side effects, and a safe therapeutic index. This compound was compared with two well-known barbiturates and will be discussed later. Derivative No. 6 is a sulfur analog of compound No. 5. The anesthetic dose of compound No. 6 is smaller than

that of compound No. 5, and its duration of action is approximately the same; but unlike compound No. 5, it produces tremors or light convulsions. The lethal dose of compound No. 6 is approximately the same as that of compound No. 5, but the therapeutic index is better. Compound No. 7 has a pentenyl and an isopropyl group on the R and R_1 positions similar to compound No. 5 but also a methyl radical in place of the hydrogen on one of the nitrogens. In comparison with compound No. 5, derivative No. 7 has a slightly smaller anesthetic dose. It, however, unlike compound No. 5, produces preanesthetic tremors. The duration of action of compound No. 7 is approximately one-third that of compound No. 5. It is also more toxic with a low therapeutic index. The anesthetic dose of compound No. 8, an isomer of compound No. 5, is approximately the same, but its duration of action is twenty minutes longer. However, it produces tremors or light convulsions. The lethal dose is smaller than that of compound No. 5 and it therefore has a smaller therapeutic index. In compound No. 9, another isomer of derivative No. 5, the isopropyl radical on the R_1 position is replaced by a *n*-propyl group. Its anesthetic dose is approximately twice that of compound No. 5. The lethal dose is such that it has a low therapeutic index and a duration of action twenty-three minutes shorter than that of compound No. 5. Compound No. 10 has a 2-pentenyl on R and an allyl on R_1. The therapeutic index is low and the duration of action, one hundred and nineteen minutes. In compound No. 11, a 2-pentenyl radical and a 1-methylpropyl radical in the R and R_1 positions, respectively, slightly lower the anesthetic dose, shorten the duration of action, and increase the lethal dose. Thus, the therapeutic index is small. The last four compounds in Table I are all isomers of compound No. 5. The double bond in the pentenyl radical, however, is on the 1 C-atom instead of the 2 C-atom. Compounds No. 13 and 14 have a secondary pentenyl radical with a methyl group and an ethyl group, respectively, on the 1 C-atom. These derivatives (Nos. 12, 13, 14, and 15) have previously been reported by Cope and Hancock (12) and by Cope, Hartung, Hancock, and Crossley (13). As indicated in Table I compounds No. 12, 14, and 15 produce preanesthetic tremors with approximately the same anesthetic and lethal doses, therapeutic index and duration of action. Compound No. 13 has an anesthetic dose and a lethal dose smaller than those of its three isomers (Nos. 12, 14, and 15), but its therapeutic index is approximately the same. The duration of action of compound No. 13 is twenty to twenty-eight minutes shorter than that of its isomers and this derivative does not produce side effects such as preanesthetic tremors.

Thus, there is no obvious relationship between the pharmacological action and chemical structure of these compounds, particularly the substitution of a pentenyl radical on one of the 5,5-positions. However, as in other barbiturates (1, 2, 3, and 6). the substitution of a methyl radical in place of the hydrogen on one of the nitrogens, an increase in the number of carbon atoms in one of the radicals, a secondary group on one of the 5,5-positions, or a sulfur atom in place of the oxygen on the 2-carbon atom, generally lowers the anesthetic dose and shortens the duration of action.

Compound No. 5 of this series appears to be the most desirable in dosage, duration of action, and therapeutic index. For further evaluation this barbiturate was compared with two well-known hypnotics, namely, Seconal® Sodium[1] (1-methylbutylallyl barbituric acid sodium salt) and Amytal® Sodium[2] (isoamyl ethyl barbituric acid sodium salt). They were administered as the sodium salt by vein to rats, rabbits and dogs; by mouth to rats in solution (sodium salt), and to dogs in capsule form. The results of the comparison are recorded in Table II. Compound No. 5, or 5-2-pentenyl)-5-isopropyl barbituric acid, is distinctly shorter acting than Seconal® Sodium or Amytal® Sodium. This duration of action is independent of the amount required to produce anesthesia, for Seconal® Sodium requires less to produce anesthesia than compound No. 5 but it was longer acting. On the average, compound No. 5 required approximately the same amount to produce anesthesia as Amytal® Sodium but Amytal® Sodium was several times longer acting than compound No. 5. Furthermore, in general, compound No. 5 appeared to have a better therapeutic index than Seconal® Sodium or Amytal® Sodium.

CONCLUSIONS

1. A series of pentenyl barbituric acid derivatives has been studied.

2. In general, the amount required to produce anesthesia is independent of the duration of action.

3. There is no obvious relationship between the pharmacological action and chemical structure of these compounds.

4. Many of these compounds are isomeric but show considerable variation in dosage, duration of action, therapeutic index, and side effects such as postanesthetic tremors, diaphragmatic spasms with tremors and preanesthetic tremors.

5. Derivative No. 5, 5-(2-pentenyl)-5-isopropyl barbituric acid, is shorter acting than either Seconal® Sodium or Amytal® Sodium, and has a better therapeutic index.

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[1] Seconal® Sodium (Secobarbital Sodium, Lilly).
[2] Amytal® Sodium (Amobarbital Sodium, Lilly).

Spectrophotometric Determination of the Principal Alkaloids of Opium*

By MERTON S. DYER† and ARTHUR J. McBAY‡

The spectra of four opium alkaloids; narceine, narcotine, papaverine, and thebaine with its acid decomposition products, were determined at various *p*H values. A method of assay for samples of opium concentrates is proposed that requires less time and a smaller sample than the existing methods. Four samples of known composition were assayed by this method and the results are good for alkaloidal assays.

ALTHOUGH MIXTURES of the alkaloids of opium have been widely used for over sixty years, there have been very few attempts to assay completely these preparations.

When this type of preparation was originally introduced it was prepared by extraction of the crude drug but now it is prepared, in most countries, by mixing the five or six most abundant alkaloids of opium in the same ratio in which they occur in opium.

Morphine hydrochloride, which is usually present to the extent of fifty per cent, is the only alkaloid for which a determination is made in many countries.

The other alkaloids are determined as a group or not determined at all. Two methods of assay have been proposed for preparations of this type. In 1940 Klyachkina and Sorochinhaya (1) introduced a method for the determination of the six principal alkaloids of Pantopon®-like preparations. This method consisted of many extractions and many precipitations. In 1947 Adamson, Handskide, and Hodgson (2) proposed a method for the assay of Papaveretum. This procedure is adequate if a large sample is available, but again it requires a great deal of time to carry out the numerous extractions and crystallizations.

The assay of the "British Pharmaceutical Codex" is similar to Adamson's method of assay.

The purpose of this investigation was to develop a method of assay that would require less time and require a smaller sample.

An analysis based upon spectrophotometry should eliminate all crystallizations and lessen the number of separations. The amount of two alkaloids may be determined simultaneously by the use of two-component spectrophotometric analysis.

* Received September 27, 1954, from the Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy, Boston.

Presented to the Scientific Section, A. PH. A., Boston meeting, August 26, 1954.

† Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Merton S. Dyer in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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Chemicals.—The alkaloids used in this investigation were commercially available at the time this investigation was undertaken with the exception of narceine and narcotine. Lately narcotine has been made available. The alkaloids or salts of the alkaloids were used as obtained with the exception of narceine and narcotine which were purified by repeated recrystallizations from appropriate solvents. Sodium hydroxide solutions were prepared by diluting a saturated solution of sodium hydroxide with recently boiled and cooled distilled water. Samples of simulated Pantopon® were prepared by mixing the alkaloids in the following proportions:

Morphine hydrochloride	50.0%
Narcotine hydrochloride	30.0%
Papaverine hydrochloride	4.0%
Codeine hydrochloride	2.5%
Thebaine hydrochloride	2.0%
Narceine hydrochloride	1.0%
To which was added Sodium chloride	10.5%

EXPERIMENTAL

Preparations of the Spectrograms.—The spectra of the alkaloids under consideration in this investigation have been reported (3–10). With the exception of the spectrograms of morphine and codeine, reported by Clark and McBay (11), the spectra for various *p*H values have not been reported. It was necessary to study the effect of *p*H on the spectra in order to establish the optimum *p*H for the quantitation and also to find the best pairs to be used for the two-component method of analysis.

Solutions containing 20 μg./ml. of thebaine, narcotine, narceine, and papaverine were prepared at *p*H values of 2, 4, 6, 8, 10, and 12. Their absorbances were determined from 220 mμ to 330 mμ. The spectra obtained by plotting the results are shown in Fig. 1.

The spectra of thebaine were found to vary on standing and the solution turned yellow. Because of this instability of thebaine, attempts were made to convert the thebaine to more stable products. Schöpf and Borkowsky (12) state that thebaine will yield one of two products when treated with hydrochloric acid, depending upon the concentration of the acid. Dilute hydrochloric acid will produce the secondary base, thebenine; while concentrated

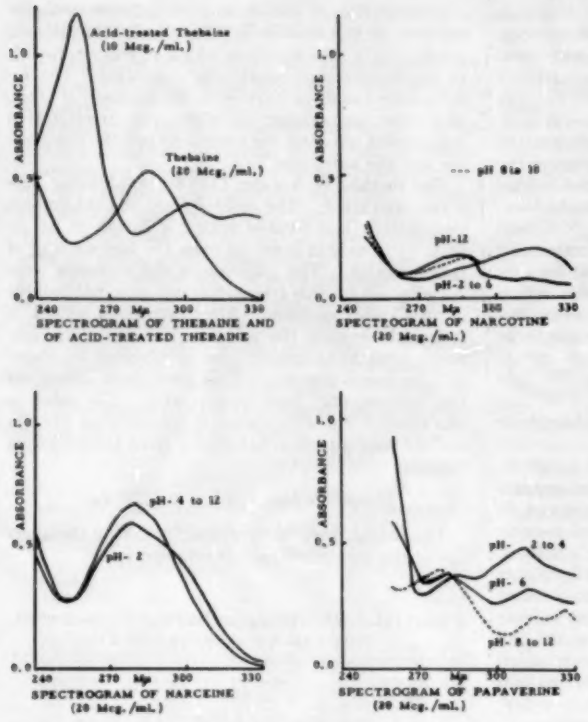

Fig. 1.—Spectrogram of thebaine, acid-treated thebaine, narcotine narcene, and papaverine.

hydrochloric acid will produce the tertiary base, morphothebaine, forming an intermediate halochromic compound that is red. Knorr (13) reported that thebaine can be partially converted into codeinone by heating with dilute sulfuric acid.

All three methods of converting the thebaine into a stable product that could be determined by the contemplated method of assay were tried. The treatment with hydrochloric acid did not yield consistent results. When the sample of thebaine was heated with 1 *N* sulfuric acid it was converted into some product or products that had a favorable spectrum for an analysis. The sample of thebaine so treated will be designated as acid-treated thebaine. The spectrum of this acid-treated thebaine is shown in Fig. 1.

Separation Procedure.—After the spectrograms had been prepared attempts were made to separate the alkaloids by means of various solvents into pairs that could be determined by two-component analysis.

Adamson and others (2) proposed a method for the separation of the alkaloids of Papaveretum by the initial extraction of an acidified solution with chloroform. Papaverine, narcotine and part of the thebaine are separated from the codeine, morphine, and narceine. This extraction affords a means of separating the sample into two portions each of which could then be re-extracted to separate further the alkaloids.

Determination of Morphine and Narceine.—The aqueous portion remaining after the extraction of the papaverine, narcotine, and thebaine contains all of the codeine, morphine, narceine, and a portion of the thebaine. Numerous attempts to determine directly the concentration of codeine and morphine were unsuccessful because of the small amounts of thebaine and narceine that were present. Caspari (14) reported that codeine could be separated from morphine and narceine by extraction with benzene from an alkaline solution. Solutions containing known amounts of narceine and codeine; morphine and codeine; and thebaine and codeine were prepared. Each of these solutions was extracted and the completeness of the extractions was determined. The solution containing thebaine and codeine was completely extracted by this procedure. An aqueous solution that would contain only morphine and narceine was obtained. Since the maximum absorbance of the morphine and narceine occur at different wavelengths in *p*H 12 solutions it was possible to determine the concentrations of the alkaloids by means of the two-component method.

The relationship between the concentration and the absorbance was determined at 278 mμ and 300 mμ for both morphine and narceine.

From the averages of Table I it was possible to establish the following equations by the method of determinants:

$$\text{Morphine (mg./ml.)} = 0.1566\,A_{300} - 0.06356\,A_{278}$$
$$\text{Narceine (mg./ml.)} = 0.04074\,A_{278} - 0.02610\,A_{300}$$

Determination of Codeine and Thebaine.—It was necessary to remove the codeine and any thebaine present from the benzene before their con-

TABLE I.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE (*p*H 12)

Conc., μg./ml.	278 mμ Abs.	278 mμ Conc./Abs.	300 mμ Abs.	300 mμ Conc./Abs.
		Narceine		
30.00	0.985	30.46	0.461	72.12
27.00	0.920	29.35	0.389	69.41
24.30	0.828	29.35	0.350	69.43
21.87	0.739	29.59	0.311	70.32
19.68	0.661	29.77	0.284	69.30
17.71	0.595	29.76	0.246	71.99
	Av.	29.72	Av.	70.43
		Morphine		
50.00	0.289	173.0	0.450	111.1
45.00	0.254	177.2	0.402	111.9
40.50	0.241	168.1	0.371	109.2
36.45	0.214	170.3	0.334	109.1
32.80	0.193	169.9	0.299	109.7
29.62	0.174	170.2	0.274	108.1
	Av.	171.5	Av.	109.9

centration could be determined. At first it was hoped that the benzene could be removed by evaporation but the residue would not dissolve completely in dilute acids. The most satisfactory method of removing the alkaloids from the benzene consisted of extraction with a dilute mineral acid. After extraction the solution was diluted and the absorbance determined. From the absorbance the concentration of codeine was calculated. This gave good results on solutions of codeine and morphine but with solutions of codeine, morphine, and thebaine, the results were inconsistent, particularly if the samples had been allowed to stand for any length of time. The solutions had a color which was similar to the yellow color produced when thebaine was treated with hydrochloric acid. Since thebaine had undergone a partial change an attempt was made to convert all of the thebaine into this colored product. Numerous samples of thebaine were treated with concentrated hydrochloric acid and also with 1 *N* sulfuric acid. The treatment with 1 *N* sulfuric acid gave the most consistent results and was found to have no effect upon samples of codeine. This acid treatment was adopted in the determination of the concentration of codeine and thebaine. After extraction with 10% sulfuric acid the sample was diluted with an equal volume of water and heated for thirty minutes on a water bath. It was then diluted to a convenient volume and the absorbance determined at 256 mμ and 285 mμ. The relationship between the concentration and the absorbance was determined at 256 mμ and 285 mμ for both codeine and thebaine.

TABLE II.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE (*p*H 2)

Concn., μg/ml.	256 mμ Abs.	256 mμ Concn./Abs.	285 mμ Abs.	285 mμ Concn./Abs.
		Codeine		
50.00	0.189	264.6	0.285	175.4
45.00	0.169	266.3	0.254	177.2
40.50	0.153	264.7	0.231	175.3
36.45	0.142	256.7	0.212	171.9
32.80	0.121	271.1	0.187	175.4
29.62	0.109	271.7	0.169	175.3
	Av.	265.9	Av.	175.1
		Thebaine Acid-Treated		
10.00	1.17	8.547	0.286	34.96
9.00	1.08	8.333	0.271	33.12
8.10	0.962	8.420	0.239	33.89
7.29	0.870	8.379	0.219	33.29
6.56	0.782	8.389	0.198	33.12
5.90	0.708	8.333	0 174	33.11
	Av.	8.40	Av.	33.73

From the averages of Table II the following equations were established:

$$\text{Codeine (mg./ml.)} = 0.2095\ A_{285} - 0.0516\ A_{256}$$
$$\text{Thebaine (mg./ml.)} = 0.01005\ A_{256} - 0.006617\ A_{285}$$

Determination of Narcotine, Papaverine, and Thebaine.—The narcotine, papaverine, and the thebaine which are in the chloroform that was used for the initial extraction had to be separated still further.

Evaporation of the chloroform solution and dissolution of the residue in dilute hydrochloric acid resulted in a solution from which it was impossible to precipitate consistently the narcotine. The results were found to vary with the amount of alkali used, the temperature at which the precipitation was carried out, and the ratio between the papaverine and the narcotine.

The method of Anneler (15) for solubilizing narcotine was tried. The residue from the chloroform evaporation was treated with a solution of potassium hydroxide in order to open the lactone ring of the narcotine. The papaverine and thebaine were not affected by this treatment and were not soluble in an alkaline medium. After extraction with 1 *N* sodium hydroxide the solution was made strongly acidic with hydrochloric acid and heated to about 90° for forty minutes. This procedure converted the "narcotinate" back to narcotine. The solution was diluted, the absorbance determined at 312 mμ and the concentration calculated from the following equation:

$$\text{Narcotine (mg./ml.)} = 0.1070\ A_{312}$$

The constant was determined by taking the average of the concentration/absorbance value.

TABLE III.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE (*p*H 2)

Concn., μg/ml.	312 mμ Abs.	312 mμ Concn./Abs.
	Narcotine	
30.00	0.278	107.9
27.00	0.251	107.6
24.30	0.224	108.5
21.87	0.204	107.2
19.68	0.188	104.7
17.71	0.167	106.0
	Av.	107.0

After the narcotine had been separated it was necessary to separate the papaverine and any thebaine present from the benzene that was used as a solvent in the treatment of the narcotine. The benzene was removed by very careful heating on a water bath. The gummy residue that resulted would not dissolve in dilute hydrochloric acid but it was found that it would dissolve readily in absolute alcohol from which it could be recrystallized in a crystalline form that would then dissolve in the dilute hydrochloric acid.

Attempts were made to calculate the concentration of both papaverine and thebaine. The inconsistencies of the results were probably due to the same change here with the thebaine as was experienced in the determination of the codeine. The same treatment of heating the solution in the presence of 1 *N* sulfuric acid was tried and the results on solutions of known concentration were good.

In order to establish the equations for the calculation of papaverine and thebaine concentrations it was necessary to determine the concentration/absorbance value at the wavelengths of 285 mμ and 312 mμ.

From the averages of Table IV it was possible to establish the following equations:

Papaverine (mg./ml.) = $0.3633\ A_{312} - 0.4083\ A_{285}$
Thebaine (mg./ml.) = $0.3033\ A_{285} - 0.2398\ A_{312}$

From the method of extraction and the equations previously established the following method of assay is proposed.

Method of Assay.—Dissolve about 150 mg. of sample, accurately weighed, in 15 ml. of water. Transfer the solution to a 150-ml. separator with the aid of three 5-ml. portions of water. Render the solution acid by the addition of one drop of dilute hydrochloric acid. Extract the solution first with 30 ml. of chloroform, then with three 20-ml. portions of chloroform. Wash each chloroform extract first with 10 ml. of 1 *N* sodium hydroxide, contained in a second separator, and finally with 10 ml. of water contained in a third separator. Filter the chloroform extracts into a dry beaker through a filter moistened with chloroform. Reserve the chloroform extracts for the determination of papaverine, thebaine, and narcotine.

TABLE IV.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE (*p*H 2)

Conc., μg/ml.	285 mμ Abs.	285 mμ Conc./Abs.	312 mμ Abs.	312 mμ Conc./Abs.
		Papaverine		
50.00	0.990	50.51	1.25	40.00
45.00	0.885	50.85	1.12	40.18
40.50	0.796	50.88	1.01	40.10
36.45	0.720	50.63	0.900	40.50
32.80	0.632	51.90	0.800	41.00
29.62	0.572	51.78	0.728	40.69
	Av.	51.09	Av.	40.4
		Thebaine Acid-Treated		
10.00	0.286	34.96	0.326	30.67
9.00	0.271	33.12	0.302	29.80
8.10	0.239	33.80	0.269	30.11
7.29	0.219	33.29	0.242	30.12
6.56	0.198	33.13	0.222	29.55
5.90	0.174	33.11	0.198	29.80
	Av.	33.73	Av.	30.01

Determination of Codeine, Thebaine, Morphine, and Narceine.—Transfer the alkaline solution in the second separator to the aqueous portion in the first separator, washing it first with the water in the third separator, then with 5 ml. of water. Render the solution alkaline to litmus by adding 1 *N* sodium hydroxide and make three five-minute extractions with 20-ml. portions of benzene. Wash each benzene extract with the same 5-ml. portion of water. Reserve the benzene layer for the determination of codeine and thebaine.

Morphine and Narceine.—Combine the aqueous portions and acidify with 10% hydrochloric acid. Dilute with water to 75 ml. and warm to insure complete solution. Cool and dilute with water to exactly 100 ml. Mix well and remove exactly 10 ml. of this solution and dilute with water to about 75 ml. Adjust the solution to a *p*H of 12, to Hydrion paper narrow range, with 1 *N* sodium hydroxide solution, carbonate-free, and dilute with water to exactly 100 ml. Determine the absorbance at 278 mμ and 300 mμ, using a sodium hydroxide blank. The following equations are used for the calculation of the concentration of morphine and narceine:

Morphine (mg./ml.) = $0.1506\ A_{300} - 0.06356\ A_{278}$
Narceine (mg./ml.) = $0.04074\ A_{278} - 0.02610\ A_{300}$

Codeine and Thebaine.—Filter the benzene extract into a clean separator through a filter moistened with benzene and extract for five minutes with each of two 15-ml. portions of 10% sulfuric acid. Combine the acid extracts, add 30 ml. of water and heat on a water bath for thirty minutes. Cool and dilute with water to 100 ml. and determine the absorbance at 256 mμ and at 285 mμ. The following equations are used for the calculation of the concentration of codeine and thebaine:

Codeine (mg./ml.) = $0.2095\ A_{285} - 0.05160\ A_{255}$
Thebaine (mg./ml.) = $0.01005\ A_{285} - 0.006617\ A_{255}$

Narcotine, Papaverine, and Thebaine-Narcotine.—Evaporate the chloroform extracts to dryness, avoiding excess heat. Dissolve the residue in 5 ml. of benzene, and then add 5 ml. of 10% alcoholic potassium hydroxide solution. Allow the solution to stand for forty minutes at 20°. Transfer the solution to a small separator with the aid of three 5-ml. portions of benzene and one 10-ml. portion of 1 *N* sodium hydroxide solution. Allow the two layers to separate and draw the lower layer into a small flask. Extract the benzene layer in turn with two 5-ml. portions of 1 *N* sodium hydroxide followed by two 5-ml. portions of water. Combine the aqueous portions and add 5 ml. of concentrated hydrochloric acid. Heat the acid solution at 95° for thirty minutes on a water bath. Cool, and dilute the solution with water to exactly 100 ml. Mix and remove exactly 10 ml. and dilute with water to exactly 100 ml. Determine the absorbance at 312 mμ, using dilute hydrochloric acid as the blank. The following equation is used in the calculation of the concentration of narcotine:

Narcotine (mg./ml.) = $0.1070\ A_{312}$

Papaverine and Thebaine.—Evaporate the benzene layer to dryness, avoiding excess heat, and dissolve the oily residue in 5 ml. of absolute alcohol. Evaporate the alcoholic solution to dryness and dissolve the residue in 15 ml. of 10% sulfuric acid. After the addition of 15 ml. of water, heat the solution for thirty minutes on a water bath. Then cool the solution and dilute it with water to exactly 100 ml. Mix the solution well and remove exactly 10 ml. Dilute the solution with water to exactly 50 ml. Determine the absorbance of the solution at 285 mμ and 312 mμ with dilute sulfuric acid as the blank. The following equations are used in the calculation of the concentration of papaverine and thebaine:

Papaverine (mg./ml.) = $0.3633\ A_{312} - 0.4083\ A_{285}$
Thebaine (mg./ml.) = $0.3033\ A_{285} - 0.2398\ A_{312}$

The concentration of thebaine in this fraction is added to that in the other fraction to obtain the total concentration of thebaine in the sample.

Results of Four Assays.—A sample of simulated Pantopon® was prepared with the alkaloids in approximately the same ratio in which they occur in Pantopon® and other preparations of this type. Samples of this simulated Pantopon® were assayed

according to the method proposed in this investigation. The results of these assays are found in Table V.

TABLE V.—FOUR ASSAYS OF SIMULATED PANTOPON®

Alkaloid	Composition of Known, %	Composition Found, %			
Morphine	41.2	38.1	38.1	39.5	39.4
Narcotine	23.8	21.9	22.8	23.8	22.6
Papaverine	3.83	2.82	4.50	3.86	2.61
Codeine	2.40	2.04	2.34	2.55	2.50
Thebaine	2.05	0.53	0.31	2.71	2.25
Narceine	0.89	[a]	1.01	0.89	[a]

[a] It was not possible to calculate the concentration from the values obtained.

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Some Pharmacological Properties of Tricyclamol, a New Anticholinergic Agent*

By ARTHUR G. ZUPKO and LEON D. PROKOP

The results of quantitative determinations of sweat and saliva before and after drug or placebo administration are reported. The effects of prolonged administration and manifestations of parasympathetic inhibition with the drug in normal and hidrose subjects are also reported. Measurement of locomotor activity in rats to ascertain the central effects of tricyclamol is discussed.

FOR MANY YEARS clinicians have recognized the fact that numerous gastrointestinal disorders result from excessive parasympathetic activity. The anticholinergic agents have been found to be clinically useful in these disease entities because of their ability to block the action of acetylcholine at ganglionic synapses and postganglionic parasympathetic nerve endings. This same blocking action, however, has also limited their usefulness in gastrointestinal disorders through absence of selective activity, since dysuria, mydriasis, tachycardia, and xerostomia also result from interruption of cholinergic transmission with these drugs. The continued research effort on antichlolinergic drugs is being directed toward uncovering more selective parasympathetic depressants.

The ideal anticholinergic agent, when used to combat gastrointestinal disorders, may be characterized as a compound possessing a high therapeutic index, a specificity of action to obviate undesirable effects, and yet is devoid of tolerance development. When ingested orally it should be capable of inhibiting gastric secretory and motor functions for many hours. None of the currently available anticholinergic compounds meets all these criteria.

Previous studies (1–4) have indicated that the suppression of sweating and salivary flow may be in direct ratio to the incidence of undesirable effects to be anticipated in continued anticholinergic therapy. Beckman (5) has reported that certain anticholinergic agents may cause stimulation of the higher centers. In view of these reports it appeared efficacious to investigate the anhydrotic, antisialogogue, and central effect of a recently introduced anticholinergic compound, tricyclamol (1-cyclohexyl-1-phenyl-3-pyrrolidino-1-propanol methyl sulfate). Such a study would give an indication as to the selectivity of cholinergic blocking action of this new quaternary amine compound.

EXPERIMENTAL

Methods.—Test groups of normal and hidrose subjects were used for quantitative determinations of sweat on various parts of the body prior to and after drug or placebo administration. The subjects were exposed to thermostatically controlled infrared heating panels mounted in a constant temperature room. Experiments were conducted at consistently high dry bulb temperatures of 92–99° F. with relative humidity variations of 41–76%. For the collection of sweat a method previously described was utilized (2), the amount of sweat secreted during a test period being represented by the change in weight of a prepared unit after removal from a body

* Received August 27, 1954, from the Department of Physiology and Pharmacology, St. Louis College of Pharmacy and Allied Sciences, St. Louis 10, Mo.
Marketed as Elorine® Sulfate (Eli Lilly and Co.) and Tricoloid® (Burroughs Wellcome and Co.).
The authors wish to thank Dr. Donald S. Searle (Burroughs Wellcome and Co.) and Dr. James B. Hammond (Eli Lilly and Co.) for drug supplies and financial support.

area. A dose of 100 mg. of tricyclamol, equivalent to twice that of the recommended single oral dose, was given to each subject.

In order to evaluate further the anhydrotic effect of tricyclamol, as well as to ascertain side effects resulting from continued ingestion of the drug, 75 hidrose and 6 normal subjects were administered the drug in varying dosages over periods ranging from six weeks to four months. In general, the routine used was similar to that previously described (3).

Double blind studies, in which neither operator nor subject knew the identity of the preparation used, were conducted throughout the course of these experiments.

The parotid salivary output of 143 volunteer males was determined before and at half-hour intervals after administration of the drug. The procedure of inducing and collecting saliva from Stenson's duct has been described in a previous report (4). An oral dose of 100 mg. of tricyclamol was used. An additional 21 subjects were used to study the effect of placebo administration on salivary flow.

Locomotor activity of albino rats was used as a measurement of the central effect of the drug. Measurement of this activity was accomplished by means of Wahmann vertically revolving drums based on a technique described by several investigators (6–8). Boyd and his associates have had success in utilizing this technique for determining the central effects of various antihistamines and chlorpromazine (9, 10).

Forty male albino rats of the Sprague-Dawley strain, weighing 200 to 250 Gm., were divided into two equal groups. The rats were fasted for seventy-two hours to augment their locomotor activity, which was recorded as revolutions of the drum/rat/hour. One group was injected subcutaneously and the second group was used for administration by mouth of varying doses of tricyclamol. Locomotor activity was measured for two hours before and four hours after drug administration. Veeder counter readings were taken at one-hour intervals. Additional details of the procedure used have been reported in a recent paper by Baird and Boyd (9).

TABLE I.—AVERAGE PERCENTAGE REDUCTION OF SWEAT IN NORMAL AND HIDROSE SUBJECTS AFTER ORAL ADMINISTRATION OF 100 MG. TRICYCLAMOL

Area of Body	Minutes after Ingestion 30	60	90	120
Dorsum of hand	9.4	22.2	29.7	25.5
Forearm	7.0	19.3	21.3	20.7
Upper arm	11.8	16.1	15.8	13.9
Forehead	16.4	27.9	34.8	36.3
Dorsum of foot	11.7	18.5	20.9	17.4
Leg	10.9	15.9	26.2	14.5
Thigh	8.8	11.4	19.3	12.8

The experiments were done during the spring and summer months with room temperatures from 21.9 to 27.3° C., relative humidity from 31.8 to 53.4%, and barometric pressure from 749 to 758 mm. of mercury.

RESULTS

Quantitative Estimation of Sweat.—One hundred and seventy-three quantitative determinations of sweat on seven different areas of the body were performed. Approximately 60% of the subjects were hidrose. There was no significant difference between the response of normal and hidrose subjects. The effect of tricyclamol in reducing sweat on various body parts is shown in Table I. From these data it appears that tricyclamol reaches a point of effectiveness somewhere between thirty and sixty minutes and a peak of effectiveness between sixty and ninety minutes. There was no further appreciable reduction of sweat beyond two hours after drug administration. The duration of action was approximately four and one-half to five hours. The average reduction of sweat was greatest on the forehead and dorsum of the hand and least on the upper arm and thigh.

Under the same experimental conditions, quantitative determinations of sweat were performed on 15 hidrose and normal subjects who were administered placebo preparations. Reduction of sweat ranged from 1.3 to 6.7% in 7 subjects but was increased from 1.7 to 7.1% in the other 8 subjects.

Effect on Hyperhidrosis.—Seventy five hidrose subjects, many of whom were used in previous studies of this type, were arbitrarily classified as individuals with mild, pronounced, or severe hyperhidrosis, depending upon the severity of symptoms. A second group of six normal subjects was used as a control. The pertinent data on the test subjects, including the scale of dosages utilized, are shown in Table II.

Fifty-seven, or 76%, of the hidrose subjects in the test group claimed effective control of hyperhidrosis. Of 38 hidrose subjects classified as mild, tricyclamol was effective in 30; of 33 classified as pronounced, the drug was effective in 26; of 4 classified as severe, it was effective in only one. The optimal daily dosage of tricyclamol for control of hyperhidrosis varied in mild cases from 150 to 200 mg.; in pronounced cases from 200 to 225 mg.; in severe cases from 225 to 300 mg. Dosages up to 300 mg. daily were ineffective in controlling hyperhidrosis in 18 of the subjects under test.

Side Effects.—Subjective manifestations of parasympathetic inhibition reported by the test subjects at the dosage level used for control of hyperhidrosis are shown in Table III. These effects, usually

TABLE II.—PERTINENT DATA ON TEST SUBJECTS

Hidrose subjects	75	Hidrose dosage	
Mild	38	Mild	50 mg. t.i.d.–50 mg. q.i.d.
Pronounced	33	Pronounced	100 mg. b.i.d.–75 mg. t.i.d.
Severe	4	Severe	85 mg. t.i.d.–100 mg. t.i.d.
Normal subjects	6	Control dosage	75 mg. b.i.d.
Average age (years)	23.4	Duration of test period	6 weeks to 4 months
Sex	62 M		
	19 F		

TABLE III.—SIDE EFFECTS REPORTED BY TEST SUBJECTS AFTER CONTINUED INGESTION OF TRICYCLAMOL

Effect	Subjects Reporting Effects	
	Number	Percentage
Constipation	3	3.7
Headache	5	6.2
Dysuria	1	1.2
Heartburn	2	2.4
Xerostomia	17	20.9
Mydriasis	4	4.9
Drowsiness	2	2.4

of a mild and transient character, were reported by two controls and 21 hidrosc subjects, several of whom reported more than one side effect. In two instances persistent mydriasis necessitated discontinuance of the medication. The majority of side effects were reported by subjects at a daily dosage level in excess of 200 mg.

Antisialogogue Effect.—The results of quantitative estimations of the parotid salivary output of 143 male subjects before and at half-hour intervals after oral ingestion of 100 mg. of tricyclamol are shown in Table IV. It will be noted that the maximum suppression of saliva occurs at the sixty-minute post-drug period. No further significant suppression of saliva was discernible beyond a two-hour period. Complete cessation of salivary flow was not observed in any of the subjects under test.

The deviation from normal salivary flow in the 21 control subjects administered placebo preparations did not vary more than 5.1%.

Effect on Locomotor Activity.—Each group of animals was divided into subgroups of four. One subgroup served as a control while the remaining groups received a specified dose of tricyclamol. The experiments were repeated at weekly intervals and the subgroups rotated in a crossover until each animal had received identical rotation treatment.

Tricyclamol was administered orally in doses of 150 to 450 mg./Kg. and subcutaneously in doses of 50 to 250 mg./Kg. Within this dosage range we were unable to demonstrate a significant stimulation of higher centers under the experimental conditions stated. Beyond this dosage range, evidence of central stimulation was observed in the form of convulsions. Unfortunately, recording of locomotor activity at these dosage levels was impossible because of the fact that either the rats died shortly after convulsing or locomotion was prevented, presumably because of an ensuing paralysis of motor impulses to skeletal muscles.

As noted in Table V, some degree of depression was noted at the lower dosage levels. Doses up to and including 250 mg./Kg. administered orally had a consistently significant (P less than 0.15) depressive effect as did doses up to and including 100 mg./Kg. subcutaneously. No significant changes in locomotor activity were observed at the higher dosage levels used.

TABLE V.—EFFECT OF TRICYCLAMOL ON LOCOMOTOR ACTIVITY

Method of Administration and Dosage (mg./Kg.)	Average Number of Rev./Rat/Hr.	Average S.E.
Oral		
0	218	21
150	133	19
250	111	18
350	187	22
450	226	24
Subcutaneous		
0	206	19
50	112	17
100	137	20
150	221	24
200	214	23

TABLE IV.—EFFECT OF TRICYCLAMOL ON HUMAN PAROTID SALIVARY FLOW

Drug and Dosage	Number Tested	Pre-Drug Saliva Collected	Post-Drug Saliva Collected				Maximum % Salivary Suppression
			30 Minutes	60 Minutes	90 Minutes	120 Minutes	
Tricyclamol 100 mg.	143	1.38[a, b] (0.61–6.27)	1.29 (0.54–6.12)	1.01 (0.09–4.73)	1.08 (0.13–4.64)	1.12 (0.10–5.27)	26.81
Controls (placebo)	21	1.31 (0.74–5.93)	1.33 (0.66–6.21)	1.27 (0.81–5.77)	1.24 (0.68–5.91)	1.29 (0.70–6.88)	...

[a] Each sample collected over a five-minute period.
[b] Values not in parenthesis represent average volume in ml. Figures in parenthesis represent the range in volume obtained.

DISCUSSION

The degree of salivary and sweat suppression as well as incidence of side effects occurring with administration of tricyclamol compares favorably with that of other anticholinergic agents studied under similar experimental conditions (1–4). It is apparent, however, that tricyclamol fails to meet the requirements of an ideal anticholinergic agent. Nevertheless, continued progress is being made toward attainment of this objective as evidenced by the fact that, although lacking in specificity, tricyclamol does appear to possess a high therapeutic index and seems to be devoid of tolerance development. It has also been reported to be capable of inhibiting gastric secretory and motor functions for many hours when ingested orally (11).

Placebo administration to subjects throughout both sweat and salivary experiments served to ascertain the accuracy of the experimental methods used and to substantiate subjective reports of side effects. The authors were particularly fortunate in being

able to utilize two different placebo forms identical in appearance to the commercially available products: a pulvule form (Elorine® Sulfate, Eli Lilly and Co.) and a sugar-coated compressed tablet (Tricoloid®, Burroughs Wellcome and Co.). Thus the interchange of pulvule and tablet or pulvule placebo and tablet placebo tended to reduce the weight of the psychological factor inherent in all placebo-controlled studies.

The depression of locomotor activity in rats by low doses of tricyclamol may conceivably account for the drowsiness reported by some subjects undergoing anticholinergic therapy. In our opinion, the degree of depression exhibited is not sufficient to warrant discontinuing the practice of combining these agents with a sedative component, particularly in peptic ulcer therapy.

Since acetylcholine is the chemical mediator of nerve impulses to skeletal muscles, it is logical to assume that large doses of tricyclamol effectively block the effect of acetylcholine at these sites and prevent locomotion in the rat. In our experience, only toxic doses will produce central stimulation in rats.

SUMMARY

1. One hundred and seventy-three quantitative determinations of sweat on seven different areas of the body were performed before and after tricyclamol administration. Suppression of sweating was greatest on the forehead and dorsum of the hand and least on the upper arm and thigh.

2. Under the experimental conditions stated, it appears that tricyclamol reaches a point of effectiveness somewhere between thirty and sixty minutes and a peak of effectiveness between sixty and ninety minutes. The duration of action was four and one-half to five hours.

3. Tricyclamol effectively controlled hyperhidrosis in 76 per cent of a group of 75 hidrose subjects administered the compound in varying doses for six weeks to four months. Dosages up to 300 mg. daily were ineffective in controlling hyperhidrosis in 18 subjects.

4. Quantitative estimations of the parotid salivary output of 143 volunteer males were performed before and at half-hour intervals, after ingestion of 100 mg. tricyclamol. The maximum percentage of salivary suppression, which occurred at the sixty minute post-drug period, was 26.8 per cent.

5. The anhydrotic and antisialogogue actions of tricyclamol compare favorably with those of other anticholinergics studied under similar experimental conditions.

6. Subjective manifestations of parasympathetic inhibition, substantiated by placebo administration and double blind studies, were reported by two controls and 21 hidrose subjects. A majority of the side effects were reported by subjects at a daily dosage schedule in excess of 200 mg. Only two subjects were forced to discontinue the medication due to persistent mydriasis.

7. Under the experimental conditions stated, low doses of tricyclamol inhibit locomotor activity in the rat. It would appear that only toxic doses produce central stimulation.

8. Tricyclamol fails to meet certain arbitrary requirements of an ideal anticholinergic agent. Nevertheless, continued progress is being made toward attainment of this objective.

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals

Urushiol
Veratridine
Conessine
Guanidine hydriodide
Sphingosine
Methoxyconiferine (Syringin)
Traumatic acid
Deoxyglucose
Allocholesterol (Coprostenol)
Bismuth ethyl camphorate
Cysteine sulfenic acid
5,7-Dihydroxyflavone (Chrysin)
Aloetic acid
Dulcitan
L-Galactonic acid
Ricinine
Cellotriose
Glutaconic dialdehyde
Delta valerolactone
Lecithinase

A Study of the Reproducibility of Viscosity Determinations Using Gelatin Sols*

By LEWIS C. LAPPAS† and EDGAR P. KING‡

A statistical study was conducted with the cooperation of several manufacturers of pharmaceutical gelatins. Common samples of bone, porkskin, and calfskin gelatins were distributed to the participating laboratories for the determination of viscosity. The gelatin viscosity testing procedure established by the National Association of Glue Manufacturers was used to determine the initial viscosity and the corresponding viscosity of these gelatins after heating 17 hours at 60°. The purpose of this study was to estimate the reproducibility of viscosity, using the established test procedure, for each of the three types of gelatin. The specific objective, for each type of gelatin, was to estimate the relative magnitudes of three types of variations as follows: (1) Variation within analyst; the variation in the results of a single analyst using a given set of equipment; (2) Variation within laboratory; the variation between the results of different analysts using the same equipment; and (3) Variation between Laboratories; the variation between results of different analysts using different sets of equipment.

GELATIN is a high molecular weight protein of the emulsoid type which is prepared according to the United States Pharmacopeia XIV by the acid or alkaline extraction of its precursor derived from" . . . the skin, white connective tissue, and bones of animals . . . ." It is conceivable, under these conditions, that many closely related proteins could be produced to yield gelatins which are not identical in their chemical and physical properties. The inconsistencies of such a product are encountered when one attempts to establish chemical and physical specifications to control the purchase of various grades and blends of gelatin.

The viscosity of gelatin sols represents one physical property which must be controlled for the uniformity of thickness of cast films in the manufacture of the two-piece telescoping capsule and the soft elastic capsule. It also serves as a measure of the degree of thermal degradation over a period of time. However, viscosity determinations obtained from the same sample under specified conditions show considerable variation. The magnitude of this variation appears to depend on whether the test results are obtained by a single analyst, by different analysts in the same laboratory, or by analysts in different laboratories. This study was conducted to estimate the reproducibility of the viscosity as determined by a specified procedure for three types of gelatin: bone, porkskin, and calfskin. The specific objective for each gelatin was to estimate the relative magnitudes of three types of variation: (*a*) variation within analysts; the variation in the results of a single analyst using a given set of equipment; (*b*) variation between analysts; the variation in the results of different analysts using the same equipment; (*c*) variation among laboratories; the variation in the results of different analysts using different sets of equipment

The viscosity of gelatin sols, which represents the internal friction or resistance to flow, is usually measured with a calibrated capillary pipet in the gelatin industry. The capillary pipet permits the application of Poiseuille's Eq. (1) for simple or Newtonian liquids, i. e., those liquids whose rate of shear is proportional to the shearing stress. Some of the factors which induce many liquids to behave in a non-Newtonian or complex manner are nonhomogeneity at low temperatures, decomposition at high temperatures, molecular asymmetry, and instability at a constant or increasing shear rate. This complex behavior may be detected readily in a capillary pipet by measuring the viscosity of a liquid in several viscometers of different capillary diameters, and in a rotational viscometer by varying the rotational speeds. In either instance a Newtonian liquid would exhibit a constant viscosity with a change in the rate of shear. Frequently, the control of certain conditions such as concentration, temperature, degree of particle dispersion, and rate of shear will cause a liquid to be within a Newtonian range. Various investigators (2, 3) have demonstrated this phenomenon in gelatin sols by the control of temperature and concentration. Since it appeared that gelatin sols could be simple in behavior, Croome (4) studied the variation of viscosity with temperature in dilute sols of gelatin and represented this variation by a simple

* Received August 27, 1954, from Eli Lilly and Co., Indianapolis, Ind.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
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formula developed by Andrade (5) where $v = Ae^{K/T}$, v being the viscosity, T being the absolute temperature, and A and K being constants for the liquids used. The plot of the logarithm of the viscosity against the reciprocal of the absolute temperature is then a straight line.

This formula was applied to each of the three types of gelatin used in this study at 12.5% w/w concentration in the temperature range of 45 to 60°. A Ubbelohde suspended level viscometer was used to determine the viscosity at increments of five degrees centigrade from 60 to 45° and returning in the same manner to 60° within a period of one hour. In all instances the plot of the logarithm of the viscosity against the reciprocal of the absolute temperature produced fitted curves which were linear and had the same slope. The above experiment conducted in a temperature range of 40–60° showed a slight deviation from linearity at 40° which might be attributed to nonhomogeneity of the sol as the gel point was approached. After a heating period of one and one-half to two hours, thermal decomposition caused a departure from the straight line on the return curve. It appears that these gelatins behaved as simple liquids in the temperature range of 45–60° for a period of one hour.

It has been shown that a constant or increased rotational torque in gelatin sols will produce a typical hysteresis loop which is indicative of thixotropic materials (6). The method described by Green (7) was used to measure the thixotropic effect of the three types of gelatin used in this study in 12.5% and 25% w/w concentrations. The gelatin sols at 60° were covered with a thin film of light paraffin oil to prevent gelation and the Brookfield Model LVF viscometer was used at varying speeds of 6 to 60 r. p. m. to produce continuous up-and-down curves within a period of five minutes. In all instances there appeared to be no indication of thixotropy under the described conditions; i. e., the plot of the viscometer reading against r. p. m. with continuous up-and-down curves produced a straight line through the origin and the viscosity remained constant. This evidence is indicative of Newtonian behavior. However, it was observed with increased time at 60 r. p. m. that a gradual viscosity decrease occurred which approached complete reversal ten minutes after discontinuing the shear force. It is probable that the thixotropic effect would be more pronounced with shearing rates greater than 60 r. p. m. It appears that these gelatin sols can exhibit Newtonian behavior within a limited range of rotational force and for limited periods of time.

EXPERIMENTAL

This study was conducted with the cooperation of the analytical laboratories of several manufacturers of pharmaceutical gelatins. Common samples of three types of gelatin: bone, porkskin, and calfskin, of known densities were distributed to the participating laboratories[1] for the determination of the initial viscosity and the corresponding viscosity obtained after heating for seventeen hours at 60°. Each gelatin represented a blend of various grades of the same type of gelatin in its final composition. These gelatins were typical of normal industrial production. Each sample was ground to a fine particle size and blended individually in a twin-shell cone blender to insure uniformity. The analyses found for each of the gelatins are tabulated:

Analysis	Gelatin		
	Bone	Porkskin	Calfskin
Bloom strength, Gm.	244	265	168
Moisture, %	10.24	10.01	10.77
pH (1.5% soln.)	5.82	4.89	7.10
Sulfite as SO_2, %	Nil	Nil	Nil
Phosphate as P_2O_5, %	Trace	Trace	Trace
Total ash, %	1.89	1 12	2.40
Sulfated ash, %	1.15	0.79	1.63
Particle size			
% through 10 mesh	100	100	100
% through 100 mesh	9.6	11.1	32.9
Isoelectric point (pH)	4.8	8.3–8.5	4.9

The procedure used for the determination of the initial viscosity was essentially that adopted by the National Association of Glue Manufacturers (8). An aqueous sol of 12.5% w/w was prepared at 62° after a gelatin hydration period of six hours to overnight. The absolute viscosity, expressed as millipoises, was determined at 60° using a 100-cc. calibrated capillary pipet. This gelatin sol was collected immediately into its original container as it flowed through the pipet. The container was then stoppered tightly and placed in a constant temperature bath held at 60°. The sol was removed from the water bath after seventeen hours, the temperature was raised to 62° within five minutes, and the viscosity was determined at 60° using the same calibrated capillary pipet.

Each of the participating laboratories prepared ten individual sols in the manner described and reported their findings for the initial and the seventeen-hour viscosities for each sol. It was left to the discretion of the analyst to conduct these tests singly, as duplicates, or as groups, in keeping with his established routine.

This laboratory restandardized a previously calibrated pipet at 60° to read as absolute viscosity

[1] The writers wish to thank the following for their excellent cooperation in making this study possible: Chalmers Gelatin Corp.; The American Agricultural Chemical Co.; United Chemical and Organic Products—Division of Wilson & Co., Inc.; Vyse Gelatin Co.; Swift & Co.—Gelatin Department; Atlantic Gelatin—Division General Foods Corp.; Kind & Knox Gelatin Co.; Messrs. F. Hluchan and W. G. Richardson, Raw Material Testing Laboratory, Eli Lilly and Co., Indianapolis, Ind.; Mr. F. L. Hicks, Pharmaceutical Pilot Plants, Eli Lilly and Co.

(millipoises) according to the ASTM procedure (9) using standard viscosity oil samples *L* and *M* purchased from the National Bureau of Standards. The viscosity results obtained with the various gelatin samples and this pipet were used as the comparative controls in this study.

RESULTS

Variation within Analysts.—One of the major objectives was to estimate the precision of both the initial and seventeen-hour viscosity determinations made by a single analyst with a given gelatin sample using the prescribed test procedure. The detailed results of this phase of the study are shown in Table I. The recorded values are standard deviations (10) of individual determinations each computed from a set of ten independent measurements. Two complete sets of results are shown for those laboratories which had two participating analysts.

It is apparent that some analysts were able to obtain better agreement among their results than others. Figure 1 shows the pooled standard deviation (12) for the initial determinations of each analyst. Chance fluctuation about the middle line should exceed the width of the band between the upper and lower limit only about one time in twenty. Actually, five of the eleven points fell outside this range.

Slight degrees of variation within analysts might be attributed to the acceptable tolerances in the viscosity procedure for the gelatin concentration, the degree of hydration with time, the sol preparation time, and the inherent variation due to the mechanical blending of the gelatin. Marked degrees of variation within analysts could be attributed to operational technique which is affected by analyst technique and equipment. These differences might well be due to deviations from the given procedure such as the control of time and temperature

TABLE I.—VARIATION WITHIN ANALYSTS

	Standard Deviation of Individual Determinations					
	Initial Viscosity			Seventeen-Hour Viscosity		
Laboratory	Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin	Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin
Control	2.22	3.14	1.85	1.08	1.36	1.71
	2.25	2.46	3.02	2.33	2.80	2.24
A	1.26	1.70	1.45	2.28	2.12	1.24
B	0.82	0.68	0.53	1.79	1.55	1.35
C	1.29	3.88	1.45	2.64	2.37	2.03
D	1.27	0.68	0.77	1.18	0.89	0.91
	3.16	1.99	1.03	1.26	1.26	0.94
E	2.12	1.15	1.52	1.69	1.35	1.84
F	0.94	1.51	1.53	2.20	1.53	1.44
G	3.10	3.54	2.31	2.11	2.15	1.79
	2.08	3.53	1.84	2.11	1.64	1.06

The statistical analysis of the initial viscosity determinations showed that some groups of ten determinations were significantly more precise than others. These differences in precision were not directly related to the type of gelatin analyzed. A test for homogeneity of variance (11) was applied to the results of each analyst and only two of the eleven analysts showed appreciably different precision with one type of gelatin than with another.

for the hydration and sol preparation of gelatin, the accuracy of efflux times, and the homogeneity of the sol during the measurement of the viscosity.

The statistical analysis of the seventeen-hour viscosity determinations showed that the overall precision was not significantly different from that obtained in the initial determinations. No significant difference in precision was found for the three types of gelatin. Here, again, it was observed that

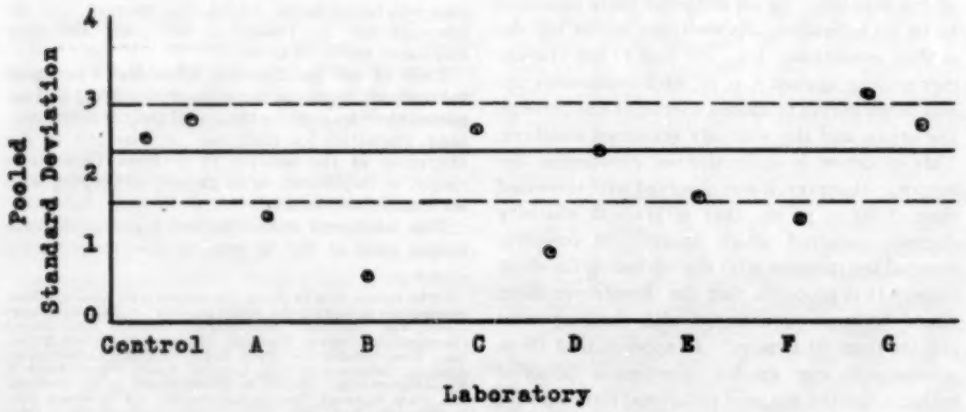

Fig. 1.—Variation within analysts

some analysts were more precise than others. It is likely that slight differences in agreement within analysts stemmed from such factors as the normal variation in the rate of thermal decomposition of gelatin and the variation due to acceptable tolerances in the given procedure. Experience has revealed that one of the greatest contributing factors to marked degrees of inconsistency in this test is the deviation of temperature during the seventeen-hour heating period. It is conceivable that the large variation observed in some instances was due to the inability to control this temperature more closely.

The standard deviations for within analyst variation when estimated from all of the initial viscosity determinations, all of the seventeen-hour viscosity determinations, and all of the results of both determinations were 2.09, 1.78, and 1.94, respectively. Since there were marked differences in precision from one analyst to another, the pooling of all of the standard deviations into a single figure does not offer the strictest degree of accuracy. However, a single figure is still of value for estimating the order of magnitude of within analyst variation. Using the pooled standard deviation of 1.94 as a measure of this variation, one would expect that two averages, each computed from ten determinations by the same analyst, should differ by approximately 0.4%.

Variation between Analysts.—The second major purpose of the study was to estimate the nature and comparative magnitude of the variation between the results of two different analysts each using the same equipment. The summary information for initial viscosities (Table II) was obtained from three participating laboratories. Each entry in the first three columns is the average of ten determinations by the first analyst minus the average of ten determinations by the second analyst. Three such differences are recorded, one for each type of gelatin. The average difference between analysts is shown in the column next to the last. shown are computed so that approximately nineteen out of twenty intervals should bracket the true difference or bias. Since all three intervals include zero, there is insufficient evidence for the existence of a bias between any of the three pairs of analysts. This does not imply that the results of different analysts show as good agreement as those of a single analyst. It simply suggests that the discrepancy between analysts fluctuates in a random manner. Wide discrepancies in reproducibility of the viscosity determinations from one analyst to another within the same laboratory indicate differing abilities of the analysts to reproduce the experimental procedure from one determination to the next. General operational technique then appears to be the most significant factor controlling the amount of variation between analysts using the same equipment.

The comparison of the variation within analysts to the variation between analysts is shown in Table III for the initial viscosity determinations. Two of the three laboratories showed considerably better agreement among the results of a single analyst than between the results of different analysts.

The corresponding analysis of the seventeen-hour

TABLE III.—COMPARISON OF VARIATION WITHIN ANALYSTS TO VARIATION BETWEEN ANALYSTS (INITIAL VISCOSITY)

Laboratory	The Standard Deviation of Averages of Ten Determinations	
	Estimated from Variation within Analysts	Estimated from Variation between Analysts
Control	0.80	2.31
D	0.54	4.00
G	0.89	0.56
(Root mean square) Average	0.76	2.69

TABLE II.—VARIATION BETWEEN ANALYSTS (INITIAL VISCOSITY)

Laboratory	Difference between Averages of Two Analysts in the Same Laboratory			Average Difference	95% Confidence Interval for the True Difference
	Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin		
Control	−5.28	0.40	−2.01	−2.30	−9.36 to 4.76
D	1.91	3.67	8.88	4.82	−4.19 to 13.83
G	0.40	1.20	0.50	0.70	−0.38 to 1.78

These average differences constitute evidence of a bias between analysts only if they are typical of the differences obtained for each type of gelatin. For example, the results of the first analyst for the control laboratory averaged 2.30 millipoises lower than the results of the second analyst. This does not necessarily mean that the first analyst consistently obtained lower viscosities for all types of gelatin. A glance at the three differences which make up the average shows that the first analyst averaged as much as 5.28 millipoises below the second analyst for porkskin gelatin, and 0.40 millipoises higher than the second analyst for calfskin gelatin. It thus appears from the results of the two analysts that an erratic fluctuation exists rather than a bias. The last column in Table II gives the results of a statistical test for the presence of bias. The confidence intervals viscosity determinations led to essentially the same conclusions. The differences between analysts (Table IV) again showed no evidence of a bias. In all three laboratories the variation within analysts was as small or smaller than the variation between analysts (Table V). Here, as in the initial viscosity determinations, operational technique appears to be the factor controlling the degree of uniformity of results between analysts using the same equipment.

Combining the evidence from the initial and the seventeen-hour viscosity determinations, the variation between analysts appeared to average from two to three and one-half times the variation within analysts. Specifically, the difference between averages obtained by different analysts in the same laboratory could be expected to range from 0.9 to 1.5% in a typical instance.

TABLE IV.—VARIATION BETWEEN ANALYSTS (SEVENTEEN-HOUR VISCOSITY)

Laboratory	Difference between Averages of Two Analysts in the Same Laboratory: Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin	Average Difference	95% Confidence Interval for the True Difference
Control	−1.51	0.45	−0.10	−0.38	−2.91 to 2.15
D	0.63	−2.16	2.20	0.22	−5.24 to 5.68
G	2.00	−1.50	−2.20	−0.57	−6.16 to 5.02

TABLE V.—COMPARISON OF VARIATION WITHIN ANALYSTS TO VARIATION BETWEEN ANALYSTS (SEVENTEEN-HOUR VISCOSITY)

Laboratory	The Standard Deviation of Averages of Ten Determinations: Estimated from Variation Within Analysts	Estimated from Variation between Analysts
Control	0.64	0.64
D	0.34	1.28
G	0.58	1.36
(Root mean square) average	0.54	1.14

Variation among Laboratories.—The final objective of the study was to estimate the variation among the determinations made by different analysts in different laboratories, and to compare this variation with the other two types discussed previously. The basic data for the initial viscosity determinations are shown in Table VI. Each entry is the difference between the average for the particular laboratory and the average for the control laboratory. The difference for each type of gelatin, the average difference, and the 95% confidence interval for the true difference are tabulated.

The confidence intervals show striking evidence that laboratory *A* obtained consistently higher results than the control laboratory, while laboratory *G* obtained consistently lower results. It is quite possible that further data would also establish that laboratories *B* and *F* would obtain results that were consistently higher and lower, respectively, than the control laboratory.

Table VII illustrates the results obtained by the control laboratory using a pipet that was purchased as one calibrated for use in the determination of gelatin viscosity. This pipet was recalibrated using a standard procedure and the results obtained by using the original efflux times, together with the new calibration constants, were established as the control averages. The two sets of results differ by as much as 10%. This suggests that the observed differences among laboratories might be reduced considerably by the use of a procedure for pipet calibration which was common to all laboratories.

The data for the seventeen-hour determinations are shown in Table VIII. The 95% confidence intervals show decisive evidence that laboratory *A* obtained consistently higher results than the control laboratory, while laboratories *B* and *G* obtained consistently lower results. The confidence intervals also suggest that additional data for laboratories *E* and *F* might reveal consistently lower results. Since the variation within analysts and among analysts for the seventeen-hour determinations was apparently small, it follows that differences in viscosity due to the variation in the rate of hydrolysis of gelatin were also small. A laboratory which reported high initial viscosities as compared to the

TABLE VI.—VARIATION AMONG LABORATORIES (INITIAL VISCOSITY)

Laboratory	Difference between Averages of Indicated and Control Laboratories: Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin	Average Difference	95% Confidence Interval for the True Difference
A	8.32	6.26	8.46	7.68	4.62 to 10.74
B	4.72	3.56	1.56	3.28	−0.69 to 7.25
C	4.07	−3.27	3.43	1.41	−8.69 to 11.51
D	0.21	3.04	−1.47	0.59	−5.07 to 6.25
E	0.16	−4.49	−2.01	−2.11	−7.89 to 3.67
F	−0.91	−1.13	−3.60	−1.88	−5.59 to 1.83
G	−3.98	−6.84	−6.19	−5.67	−9.40 to −1.94

TABLE VII.—EFFECT OF PIPET RECALIBRATION

	Analyst X: Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin	Analyst Y: Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin
Average Initial Viscosity						
Pipet 1[a]	215.19	200.40	222.13	221.09	200.43	224.31
Pipet 2[b]	202.64	189.34	208.94	207.92	188.94	210.95
Average Seventeen-Hour Viscosity						
Pipet 1	159.59	159.80	160.04	161.32	159.27	160.15
Pipet 2	152.27	152.49	152.68	153.78	152.04	152.78

[a] This pipet was purchased as a calibrated pipet intended for gelatin viscosity determinations.
[b] Pipet 1 recalibrated according to ASTM procedure D445–53T. The viscosity determinations were obtained by using the same efflux times obtained with pipet 1 and the recalculated pipet constants.

control would be expected to report high seventeen-hour viscosities. This being so, the differences in the procedure for pipet calibration would be the major variable. However, it was observed that laboratory *B* reported a high average for initial viscosity determinations and a low average for seventeen-hour viscosity determinations. Since the variation within analysts for the seventeen-hour viscosity was small, the apparent cause for such a great difference was the maintenance of a relatively constant but imprecise temperature control during the heating period. Marked differences in the seventeen-hour viscosity determination, which were not attributable to differences in pipet calibrations, could be minimized by effecting more exacting temperature controls.

Individual laboratories differed by as much as 6–9% in their average determinations of both the initial and seventeen-hour viscosities. This is approximately seven times the expected difference between analysts, and about twenty times the expected difference within analysts.

TABLE VIII.—VARIATION AMONG LABORATORIES (SEVENTEEN-HOUR VISCOSITY)

Laboratory	Difference between Averages of Indicated and Control Laboratories: Porkskin Gelatin	Calfskin Gelatin	Bone Gelatin	Average Difference	95% Confidence Interval for the True Difference
A	3.53	6.09	3.17	4.26	0.31 to 8.21
B	−8.92	−6.06	−7.23	−7.40	−10.97 to −3.83
C	−2.75	7.19	8.59	4.34	−11.02 to 19.70
D	−0.10	3.28	0.23	1.14	−3.49 to 5.77
E	−2.25	−1.97	−4.08	−2.77	−5.62 to 0.08
F	−8.96	−3.80	−6.45	−6.40	−12.81 to 0.01
G	−8.72	−7.26	−11.53	−9.17	−14.56 to −3.78

SUMMARY

1. Bone, calfskin, and porkskin gelatin sols appeared to behave as Newtonian liquids for limited periods of time under the conditions established for the viscosity testing procedure.

2. The difference between two averages of ten determinations, each obtained by the same analyst, was estimated as 0.4 per cent on the average. This figure cannot be regarded as typical of all analysts in the study, since the within analyst precision varied from one analyst to another.

3. There was no evidence that individual analysts could obtain consistently higher precision with one type of gelatin than with another.

4. There was no evidence that individual analysts could obtain consistently higher precision in initial viscosity determinations than in seventeen-hour viscosity determinations.

5. The difference between two averages of ten determinations, each obtained by a different analyst in the same laboratory, was 0.9 to 1.5 per cent on the average.

6. There was definite evidence of consistent differences among laboratories. Individual laboratories differed by as much as 6 to 9 per cent in their average determinations of both initial and seventeen-hour viscosities. The largest differences among laboratories in the determination of the initial viscosity were attributed to the variation in the procedures used in the calibration of the viscosity pipet. The variations in the method of pipet calibration and temperature control during heating were the major contributing factors toward marked differences among laboratories in the seventeen-hour viscosity determinations.

7. The viscosity changes resulting from the heating of the gelatin sols for seventeen hours at 60° indicated a uniform rate of hydrolysis.

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A Study of Antacids*

By JACK K. DALE and ROGER E. BOOTH

A number of antacids have been tested *in vitro* to determine the *p*H range in which they show buffer activity. Since the desired maximum or minimum hydrogen ion concentration may differ for various clinical conditions, the maximum *p*H attained, the speed of neutralization, the buffer capacity, and the length of activity at any desired *p*H value were studied. Twenty-four commercial products, twenty-four antacid ingredients, and eighteen investigational formulas were considered. Interaction among antacid ingredients sometimes produced new buffer compounds making prediction of *p*H difficult. Actual *in vitro* titrations are recommended for all new antacid formulations. Variation in official grade antacid materials by these tests suggests the need for further test procedures.

NUMEROUS METHODS for the *in vitro* evaluation of antacids are described in the literature (1–10). Any attempt to relate these methods to *in vivo* conditions is hindered by the lack of agreement on the *p*H range desired of an antacid. Kirsner (11), for example, claims the buffer activity should be maintained at *p*H 4 to 5.5, Fuchs (1) specifies *p*H 3 or above while Gjaldbaek (12, 13) recommends *p*H 2 or above.

Despite this apparent disagreement it is likely that all are correct since for certain clinical purposes a final *p*H range from 4 to 6 may be desirable while for others a *p*H of 2 to 3 may suffice. Many investigators have selected *p*H 3.5 as ideal since pepsin is not appreciably active at this value (1, 7, 14, 15). Others have reported that pepsin is bound or inactivated by aluminum salts (16), bismuth compounds (17), or magnesium trisilicate (18) even at low *p*H values. Antacids containing these or other pepsin inactivators might therefore be considered somewhat effective as long as the *p*H is above that of gastric juice (circa 1.5).

The search for a pleasant tasting, low cost antacid led us to study a number of experimental formulas in comparison with twenty-four marketed antacids. The commercial products were selected largely on the basis of popularity but also included products containing single ingredients or combinations which seemed worthy of investigation.

A preliminary study was conducted to determine the *p*H values obtained when various amounts of antacids were added to 0.1 *N* acid at room temperature. This method slightly modified from that proposed by Johnson and Duncan (7), serves as an excellent screen for experimental formulas. It gives the approximate *p*H value obtained with an overdose, indicates the *p*H values given by increasing doses of antacid and suggests the proper test dose.

* Received August 27, 1954, from the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.
Presented to the Scientific Section, A. PH. A. Boston meeting, August, 1954.

Our main study used the method of Fuchs (1) (cf. Procedure B) extended to *p*H 2. Our objectives were to provide data which would be useful in predicting: (*a*) the approximate amount of acid which would be neutralized to *any* selected *p*H value, (*b*) the speed of action, (*c*) the *p*H value attained with an excess of antacid present, and (*d*) the approximate time an antacid might remain active if taken at a given dose.

According to Fuchs (1) the human stomach contains the equivalent of about 50 ml. of free 0.1 *N* HCl shortly after a meal. Further, an additional 240 ml. of 0.1 *N* acid is secreted in the following two-hour period. The titration procedure based on this estimate is thus essentially the same as that of Rossett and Flexner (19) which has been shown to correlate closely with *in vivo* studies in dogs (20) and humans (21).

PROCEDURE

(A) To a series of 100-ml. samples of 0.1 *N* HCl are added increasing quantities of liquid or powdered antacids. The *p*H is determined with a Beckman model G *p*H meter after one hour of agitation.

(B) To 50 ml. of 0.1 *N* HCl is added one dose of liquid or powdered antacid. The mixture is stirred at constant speed at 37.5°. The *p*H is recorded at the end of one, three, five, and ten minutes. After each ten-minute reading 2 ml. of 1 *N* HCl are added. This step is repeated for two hours (1) or until the *p*H drops below 2.

EXPERIMENTAL

Procedure (A) was used to evaluate three series of antacids: Table I gives typical *p*H data on common trade preparations, Table II on individual antacid chemicals and Table III on experimental combinations.

Procedure (B) was used to evaluate typical commercial liquids, powders, and tablets (Tables IV and V), individual ingredients associated with antacid formulations (Table VI) and experimental antacid formulas (Table VII).

DISCUSSION OF RESULTS

Dosage.—A dosage of 10 ml. was selected for the liquid product titration studies reported so that the

TABLE I.—TYPICAL *p*H VALUES WITH PROCEDURE A ON COMMERCIAL ANTACID FLUIDS

Formula	1	2	3	4	5	6	7	8	9	10	11	12	13
Aluminum hydroxide	5.5%	6.25%	6.25%	6.6%	2.2%	5.2%	6.25%	6.25%	6.6%	...	6.0%	...	...
Magnesium trisilicate	...	...	...	...	13.0	10.0	10.0	12.5	13.3	20.0%	...	...	...
Magnesium hydroxide	...	...	...	...	...	...	...	...	...	...	2.0	...	...
Mineral oil	...	...	10.0	...	...	...	...	...	...	...	...	...	...
Aluminum phosphate	...	...	...	...	...	...	...	...	...	...	...	4.0%	...
Aluminum dihydroxyglycinate	...	...	...	...	...	...	...	...	...	...	...	...	10.0%
Dose recommended by manufacturer, ml.	10	5–10	5–10	5–10	7.5–15	5–20	15	5–10	5–10	...	5–15	15–30	5–10
Dose tested at 25°, ml.													
0	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08
2.5	1.48	1.27	1.36	1.39	1.32	1.33	1.73	1.71	1.61	1.28	1.60	1.27	1.40
5.0	3.67	1.42	1.51	1.81	1.51	1.78	3.24	3.32	1.90	1.36	2.62	1.36	2.00
7.5	4.01	1.72	2.00	3.52	...	2.62	...	3.82	3.49	...	3.95	...	2.83
10.0	4.01	2.51	3.53	3.82	2.11	3.17	3.92	4.02	3.89	1.48	4.10	1.76	3.51
15.0	4.02	3.90	3.96	3.94	3.25	3.19	4.01	4.19	3.89	1.81	4.32	2.19	3.88
20.0	4.02	...	...	...	3.61	4.12	4.21	...	...	4.49	4.82	2.42	...
30.0	...	...	...	...	4.00	4.94	4.34	...	...	6.19	...	2.49	...
*p*H with excess antacid	4.0	3.9	4.0	3.9	4.0	4.9	4.3	4.2	3.9	6.2	4.8	2.5	3.9
Clinical coefficient to *p*H													
4 (or above)	0.075	∞	∞	∞	0.3	0.2	0.15	0.1	∞	0.2	0.1	∞	∞
3 (or above)	0.05	0.15	0.1	0.075	0.15	0.1	0.05	0.05	0.075	<0.15	<0.075	∞	<0.1
2 (or above)	<0.05	<0.1	0.075	<0.075	0.1	<0.075	<0.05	<0.05	<0.075	<0.15	<0.05	0.2	0.05

effectiveness of various formulations could more easily be compared. In our dosage studies most of the commercial antacids were effective in neutralizing 100 ml. of 0.1 *N* HCl to *p*H 3.5 or above at the higher dose recommended on the label but not all were effective at the lower recommended dose. Six of thirteen of the more popular brands of liquid products did not raise the *p*H to 4 or above even with excess antacid. One failed to raise the *p*H value above 2.5.

Tablet and powder formulations were tested at commonly prescribed levels. Antacid chemicals were compared at one-gram doses and sometimes multiples of this. Table V, titrations 34 and 35, are typical results indicating doubling the dose usually more than doubles the length of activity. Tables I, II, and III list the approximate dose required to neutralize 1 ml. of 0.1 *N* HCl to *p*H 4, 3, or 2. These "clinical coefficients" are of interest not only for comparison of activity but also in clinical treatment of hyperacidity. For example, multiplying the values at *p*H 3 by the number of "clinical units" of gastric acidity found in a patient will give the dose of antacid required to neutralize gastric juice to this *p*H value. Values at *p*H 3 are preferred since this approximates the end point of the clinical test method.

Ideal Antacid.—The ideal antacid has been characterized (1, 8, 11) as: (*a*) of high-neutralizing capacity, (*b*) rapid in initial effect, (*c*) active over a long period of time, (*d*) without "acid rebound," (*e*) without undesirable laxative or constipating effects, (*f*) not absorbed from the alimentary tract, (*g*) nonirritating, (*h*) palatable, and (*i*) inexpensive. The present report is concerned chiefly with the first four properties.

Neutralizing Capacity.—The neutralizing capacity in ml. of 0.1 *N* HCl neutralized to any given *p*H value by the doses listed can be determined from Procedure (B) titrations (Tables IV–VII) by first finding the antacid formula desired and then looking for the final *p*H range wished for under it. The number of ml. of acid equivalent of 0.1 *N* HCl can then be found by following across to the left hand column listing "total ml. of acid added."

Speed of Action.—The time to reach any desired *p*H value can be read from Tables IV–VII. The time in minutes to reach *p*H 3.5 is listed at the bottom of these tables as an example of interest to those accepting this *p*H value as optimum. These values show that the majority of commercial products tested reached *p*H 3.5 or above in one to three minutes. Antacids are usually considered satisfactory in clinical use if the desired *p*H is reached in ten minutes or less.

Length of Action.—The approximate number of minutes an antacid will remain active above any *p*H value can be determined by reading the number of minutes directly from the titrations by Procedure (B). Tables IV–VII list (at the bottom) the approximate hours an antacid will remain effective above *p*H 4, 3, and 2. This is given for each antacid tested as an aid in comparing efficiency.

It will be noted from Tables I, IV, and V that a considerable variation exists among competitive products even though these have about the same active ingredients. The method of manufacture, the other ingredients present, the age of the preparation, and similar factors are probably responsible.

TABLE II.—TYPICAL *p*H VALUES WITH PROCEDURE A ON POWDERED ANTACID INGREDIENTS

Antacid Powder	Dried Aluminum Hydroxide Gel U. S. P. "A"	Dried Aluminum Hydroxide Gel U. S. P. "B"	Dried Aluminum Hydroxide Gel U. S. P. "C"	Dried Aluminum Hydroxide Gel U. S. P. "D"	Magnesium Trisilicate U. S. P. "A"	Magnesium Trisilicate U. S. P. "B"	Magnesium Trisilicate U. S. P. "C"	Colloidal Magnesium Silicate	Aluminum Silicate C. P.	Kaolin N. F.	Bismuth Subcarbonate U. S. P
Usual dose, Gm.	0.2–1.0	0.2–1.0	0.2–1.0	0.2–1.0	4.0–15.0	4.0–15.0	4.0–15.0	...	...	...	0.3–2.0
Dose tested at 25°, Gm.											
0	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.01	1.00	1.08
0.5	1.63	1.80	1.52	2.05	1.34	1.14	1.27	1.18	1.01	0.99	1.20
1.0	1.93	2.00	2.12	3.74	1.48	1.40	1.48	1.36	1.01	0.99	1.29
1.5	3.88	2.55	3.40	3.83	1.67	1.72	1.89	1.62	1.01	1.00	1.31
2.0	3.98	3.42	3.65	3.88	1.94	2.28	4.59	2.37	1.00	0.99	1.35
3.0	4.05	3.70	3.80	3.90	4.60	5.60	6.18	5.33	1.00	0.98	1.42
5.0	4.03	3.80	3.88	3.98	6.77	6.36	6.50	5.71	1.00	0.99	1.68
*p*H with excess antacid	4.1	3.8	3.9	4.0	6.8	6.4	6.5	5.7	1.0	1.0	1.7
Clinical coefficient to *p*H											
4 (or above)	0.02	∞	∞	0.05	0.03	0.03	0.02	0.03	∞	∞	∞
3 (or above)	<0.015	0.02	0.015	0.01	<0.03	<0.03	<0.02	0.03	∞	∞	∞
2 (or above)	<0.015	0.01	0.01	0.005	<0.03	0.02	0.015	0.02	∞	∞	∞

	Polyamine Methylene Resin	Dicalcium Phosphate	Bone Phosphates	Calcium Citrate	Glycine	Magnesium Oxide	Magnesium Carbonate	Calcium Carbonate	Sodium Bicarbonate	Sodium Carboxymethylcellulose	Guar Gum
Usual dose, Gm.	1	0.5–2	0.5–1.3	...	...	0.2–2	0.3–2	0.6–4	0.6–4	...	...
Dose tested at 25°, Gm.											
0	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.0
0.5	1.35	1.48	1.58	1.27	1.43	9.90	1.92	2.10	1.47	1.17	1.0
1.0	1.58	1.89	3.18	1.65	1.90	9.94	6.92	5.71	5.61	1.20	1.02
1.5	1.90	2.58	3.86	2.72	2.33	9.96	7.50	5.81	6.50	1.31	1.02
2.0	2.32	3.28	3.96	3.19	2.55	10.02	7.68	5.81	7.00	1.42	1.02
3.0	3.82	3.40	4.02	5.24	2.78	10.02	7.80	5.83	7.10	2.17	1.03
5.0	5.60	3.49	4.09	3.29	3.08	10.08	7.82	5.86	7.50	3.23	1.02
*p*H with excess antacid	5.6	3.5	4.1	3.3	3.1	10.1	7.8	5.9	7.5	1.6	1.0
Clinical coefficient to *p*H											
4 (or above)	<0.05	∞	0.03	∞	∞	<0.005	<0.011	<0.01	<0.02	∞	∞
3 (or above)	<0.03	<0.02	0.01	<0.02	0.05	<0.005	<0.01	<0.01	0.02	∞	∞
2 (or above)	<0.02	<0.015	<0.01	<0.015	0.015	<0.005	<0.01	<0.005	0.02	∞	∞

Some variations in neutralizing capacity and length of action are also noted in comparison of different lots of the same brand of antacid. Five lots of fluid antacid number 9 compared at 10-ml. doses gave no values at *p*H 4 or above but remained at *p*H 3.5 or above for twenty, ten, thirty, twenty, and ten minutes and at *p*H 2 or above for one hundred and twenty, one hundred, one hundred and ten, one hundred and ten and one hundred minutes, respectively. This is equivalent to 270, 230, 250, 250, and 230 ml. of 0.1 *N* HCl neutralized to *p*H 2.

Acid Rebound.—Staffurth (22) has recently tested 8-Gm. doses of sodium bicarbonate or magnesium trisilicate in sixteen patients without evidence of rebound secretion. While "acid rebound" may thus be a false fear it was thought desirable to find the *p*H values attained with doses of antacids up to five Gm. (or 30 ml.) per 100 ml. of 0.1 *N* HCl (Procedure A). Of all the compositions tested (Tables I–III) only magnesium oxide, magnesium carbonate, and sodium bicarbonate gave *p*H values above 7. Since the maximum *p*H of these antacids appears to be modified by other substances, it will be necessary to test each new combination containing these ingredients.

Procedure (B) can also be used to estimate the *p*H achieved with excess antacid. Here the *p*H value after ten minutes usually represents an excess since only 50 ml. of acid are combined with a full dose of antacid.

Commercial Antacids.—Table V lists the Procedure (B) titrations for eight commercial tablets (Numbers 27–34) often prescribed by doctors using the Sippy (23) treatment for ulcers. Six of these give a maximum *p*H of 7 or more. These were the most active and prolonged antacids tested, especially numbers 27 and 28. All contain magnesium oxide and/or sodium bicarbonate as might be expected. It can be determined here that bismuth subcarbonate has no appreciable buffer capacity since 0.65 Gm. added to the formula produces no effect on the maximum *p*H attained with magnesium oxide-sodium bicarbonate or calcium carbonate-sodium bicar-

TABLE III.—TYPICAL *p*H VALUES WITH PROCEDURE A ON EXPERIMENTAL FLUIDS

Formula	14	15	16	17	18	19	20	21
Aluminum hydroxide gel	6.25%	6.25%	...	6.25%	...	...	...	...
Glycine	6.25	12.5	...	...	7.6%	15.0%	...	...
Bone phosphates	...	...	...	...	10.0	...	...	...
Dicalcium phosphate	...	...	...	13.2	10.0	...	...	...
Polyamine methylene resin	...	...	...	...	...	...	20.0%	...
Methylcellulose	5.0	5.0	20.0%	...	...	1.0	5.0	...
Calcium citrate	...	...	...	...	...	20.0	...	...
Magnesium carbonate	...	...	...	...	...	...	...	1.3%
Calcium carbonate	...	...	...	...	...	...	...	15.6
Magnesium trisilicate	...	...	...	...	...	...	...	2.6
Aluminum dihydroxyglycinate	...	...	10.0	...	...	...	...	...
Dose tested at 25°, ml.								
0	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08
2.5	1.32	1.60	1.44	1.95	1.92	1.96	1.32	1.53
5.0	1.81	2.40	1.80	2.47	3.09	2.95	1.46	5.85
7.5	2.66	2.80	2.13	2.90	3.49	3.15	1.75	6.00
10.0	3.21	3.15	3.08	3.40	3.62	3.30	2.38	5.95
15.0	3.74	3.31	3.69	4.78	3.70	3.40	3.20	6.05
20.0	3.89	3.50	3.84	4.92	3.82	3.45	4.00	6.12
30.0	4.01	3.70	3.99	5.12	3.99	3.60	4.20	6.24
*p*H with excess antacid	4.0	3.7	4.0	5.1	4.0	3.6	4.2	6.2
Clinical coefficient to *p*H								
4 (or above)	0.3	∞	0.3	0.15	0.3	∞	0.2	0.05
3 (or above)	0.1	0.1	0.1	0.1	0.05	0.05	0.1	0.05
2 (or above)	0.075	0.05	0.075	0.025	0.05	0.025	0.1	0.05

TABLE IV.—TYPICAL PROCEDURE B TITRATIONS OF COMMERCIAL ANTACID FLUIDS (10-ML. DOSE)

Formula		2	22	9	4	23	10	11	12	24	25	13
Aluminum hydroxide gel (Gm.)		0.625	0.6	0.66	0.66	..	...	0.6	..	..	...	..
Magnesium trisilicate		...	1.2	1.33	..	..	2.0	..	..	..	...	..
Calcium carbonate		...	..	..	..	1.4	...	..	..	..	0.216	..
Magnesium carbonate		...	..	..	..	..	...	..	..	..	0.4	..
Bismuth subcarbonate		...	..	..	..	..	...	..	..	..	0.4	..
Glycine		...	..	..	..	0.6	...	..	..	..	...	..
Aluminum dihydroxyglycinate		...	..	..	..	..	...	..	..	..	...	1.0
Magnesium hydroxide		...	..	..	..	..	...	0.2	..	..	...	..
Sodium lactate		...	..	..	..	..	...	..	..	2.7	...	..
K, Ca, Mg lactates		...	..	..	..	..	...	..	..	0.3	...	..
Aluminum phosphate		...	..	..	..	..	...	..	0.4	..	...	..
Minutes	Total ml. 0.1 *N* HCl (Equivalent) Added											
0	50	1.07	1.06	1.07	1.07	1.0	1.09	1.09	1.00	1.09	1.07	1.07
1	50	...	4.74	3.50	1.87	5.65	1.42	3.83	2.58	4.17	...	2.76
3	50	3.84	4.83	3.59	3.32	5.80	1.89	3.98	2.61	4.20	8.70	3.62
5	50	3.85	4.59	3.67	3.68	5.91	2.39	4.10	2.65	4.20	8.86	3.75
10	50	3.87	5.09	3.75	3.76	6.14	5.68	4.51	2.68	4.20	8.87	3.86
20	70	3.52	4.11	3.64	3.67	5.94	5.25	4.09	2.47	3.97	8.32	3.62
30	90	2.67	3.74	3.54	3.63	5.85	4.38	3.85	2.38	3.78	6.32	3.50
40	110	1.84	3.41	3.47	3.56	5.58	2.21	3.73	2.31	3.62	2.25	3.00
50	130	1.48	3.21	3.38	3.49	5.70	2.08	3.65	2.26	3.48	1.44	2.77
60	150	1.30	3.02	3.20	3.39	5.55	1.69	3.58	2.22	3.30	...	2.32
70	170	1.14	2.95	3.03	3.12	5.39	1.58	3.47	2.16	3.12	...	2.10
80	190	...	2.86	2.85	2.47	5.41	1.38	3.28	2.00	2.92	...	1.62
90	210	...	2.75	2.62	1.72	5.22	...	2.90	1.82	2.66	...	1.40
100	230	...	2.61	2.39	1.39	5.24	...	2.31	1.68	2.18	...	1.22
110	250	...	2.43	2.05	1.20	4.01	...	1.92	1.54	1.59	...	..
120	270	...	2.21	1.94	1.08	2.93	...	1.69	1.43	1.28	...	..
130	290	...	2.19	..	..	2.45	...	1.49	..	..	...	..
140	310	...	1.91	..	..	2.10	...	..	..	..	...	..
150	330	...	...	..	..	1.72	...	..	..	..	...	..
Maximum *p*H attained		3.9	5.1	3.8	3.8	6.1	5.7	4.5	2.7	4.2	8.9	3.9
Minutes to reach *p*H 3.5		3	1	1	3	1	10	1	∞	1	1	3
Hours above *p*H 4		0	0.3	0	0	1.8	0.5	0.3	0	0.2	0.5	0
Hours above *p*H 3		0.3	1.0	1.2	1.2	1.8	0.5	1.3	0	1.2	0.5	0.7
Hours above *p*H 2		0.5	2.2	1.8	1.3	2.3	0.8	1.7	1.3	1.7	0.9	1.2

bonate formulations (Table V, Titrations 27–31). The addition of this material further slightly reduces the effective antacid time at *p*H 3 or above although very slightly prolongs this time at *p*H 2.

TABLE V.—TYPICAL PROCEDURE B TITRATION OF COMMERCIAL ANTACID TABLETS

Formula		27	28	29	30	31	32	33
Calcium carbonate	(Gm.)	..	..	..	0.65	0.65	0.65	..
Bismuth subcarbonate		..	0.65	0.65	0.65	..	..	0.65
Sodium bicarbonate		0.65	0.65	..	0.65	0.65	..	0.30
Magnesium oxide		0.65	0.65	0.65	..	..	..	..
Dose: Number of tablets		1	1	1	1	1	1	1
Minutes	Total ml. 0.1 *N* HCl (Equivalent) Added							
0	50	1.0	1.05	1.10	1.05	1.00	1.00	1.00
1	50	6.51	8.56	8.98	6.49	6.11	5.68	6.78
3	50	8.54	8.85	9.02	6.52	6.41	..	7.03
5	50	8.68	9.10	9.12	6.64	6.41	5.78	7.42
10	50	8.72	9.15	9.16	7.21	7.29	6.30	7.96
20	70	8.42	9.06	9.07	7.01	..	6.22	7.96
30	90	9.18	9.18	9.01	6.88	6.38	6.53	7.95
40	110	8.90	8.89	8.95	6.72	7.09	5.46	7.84
50	130	8.92	8.70	8.88	6.58	6.68	1.97	7.32
60	150	8.65	8.63	8.80	6.38	6.87	1.30	2.20
70	170	8.50	8.46	8.72	6.06	5.93	..	1.64
80	190	8.30	8.40	8.66	5.43	5.35	..	..
90	210	8.11	8.33	8.42	2.62	1.87	..	..
100	230	7.96	8.23	8.10	1.73	1.18	..	..
110	250	7.80	8.10	6.93	1.22	..	..	..
120	270	7.70	7.82	4.10	..	..	..	..
130	290	7.60	7.42	2.25	..	..	..	..
140	310	6.45	2.98	1.52	..	..	..	..
150	330	..	1.99	..	..	..	..	..
Maximum *p*H attained		9.2	9.2	9.2	7.2	7.3	6.3	8.0
Minutes to reach *p*H 3.5		1	1	1	1	1	1	1
Hours above *p*H 4		2.3	2.2	2.0	1.3	1.3	0.7	0.8
Hours above *p*H 3		2.3	2.3	2.0	1.3	1.3	0.7	0.8
Hours above *p*H 2		2.3	2.5	2.2	1.5	1.3	0.8	1.0

Formula		34		35		22	26	54	55[a]
Aluminum hydroxide gel dried powd.		...		0.65		0.324	..	0.09	
Magnesium trisilicate		0.13		0.325		0.162	..	0.150	
Calcium carbonate		0.778		...		...	0.70	0.105	
Magnesium carbonate		0.065		...		...	..	0.06	
Glycine		...		...		...	0.30	...	
Guar gum		...		...		...	..	0.10	
Oat protein		...		...		...	..	0.45	
Dose: Number of tablets		1	2	1	2	1	2	1	2
Minutes	Total ml. 0.1 *N* HCl (Equivalent) Added								
0	50	1.07	1.07	1.01	1.05	1.00	1.07	1.07	1.08
1	50	1.15	2.00	1.48	1.77	1.20	5.22	1.29	1.52
3	50	1.28	5.70	2.18	3.67	1.48	5.64	1.62	2.44
5	50	1.40	5.88	3.50	3.69	1.90	5.72	1.84	3.44
10	50	2.06	6.08	3.67	3.76	3.05	6.00	2.30	3.81
20	70	6.71	6.04	3.58	3.68	3.11	6.15	1.54	3.57
30	90	6.22	5.93	3.52	3.61	2.78	6.09	1.21	3.02
40	110	6.14	5.92	3.42	3.57	2.18	3.67	...	2.44
50	130	6.22	5.80	3.40	3.55	1.72	2.42	...	1.81
60	150	5.57	5.43	3.32	3.52	1.34	2.04	...	1.41
70	170	1.89	5.48	3.23	3.49	...	1.72	...	...
80	190	1.35	5.24	3.10	3.47	...	1.22	...	...
90	210	...	5.40	2.81	3.42	...	...	...	...
100	230	...	5.55	2.69	3.39	...	...	...	...
110	250	...	5.65	1.80	3.36	...	..	...	...
120	270	...	5.65	1.45	3.32	...	..	...	...
130	290	...	5.50	..	..	..	..	...	...
Maximum *p*H attained		6.7	6.1	3.7	3.8	3.1	6.2	2.3	3.8
Minutes to reach *p*H 3.5		20	3	5	3	∞	1	∞	10
Hours above *p*H 4		1.0	2.2	0	0	0	0.5	0	0
Hours above *p*H 3		1.0	2.2	1.3	2.0	0.3	0.7	0	0.5
Hours above *p*H 2		1.0	2.2	1.7	2.0	0.7	1.0	0.2	0.7

[a] Contains aluminum hydroxide gel dried, magnesium trisilicate, magnesium oxide, polyamine methylene resin.

Its use is then dependent on its protective or pepsin inactivating action.

Tablet product 34 (Table V) is the tablet of choice if a product is desired which doesn't exceed *p*H 6.8 regardless of dose and which will hold the *p*H above 4 for one hour with a one-tablet dose or for more than two hours with a two-tablet dose.

There is at present no liquid antacid which will

TABLE VI.—TYPICAL PROCEDURE B TITRATIONS OF POWDERED ANTACID INGREDIENTS (1-GM. DOSE)

Chemical Tested		Veegum®	Silica Gel	Bentonite	Methyl-cellulose	Sodium Cellulose Sulfate	Sodium Carboxy-methyl-cellulose	Calcium Gluconate	Calcium Glycero-phosphate
Minutes	Total ml. 0.1 *N* HCl (Equivalent) Added								
0	50	0.93	0.93	0.99	0.99	1.00	1.08	0.88	0.95
1	50	0.99	0.93	0.99	0.99	1.00	1.40	1.59	2.03
3	50	0.99	0.93	0.99	0.99	1.00	1.51	1.59	2.03
5	50	0.99	0.93	0.99	0.99	1.00	1.55	1.60	2.03
10	50	0.99	0.93	0.99	0.99	1.00	1.65	1.60	2.03
20	70	0.85	0.82	0.85	0.87	0.84	1.56	1.25	1.42
30	90	0.73	0.78	0.75	0.76	0.72	1.36	0.90	1.20
Maximum *p*H attained		1.0	0.9	1.0	1.0	1.0	1.6	1.6	2.0
Hours above *p*H 2		0	0	0	0	0	0	0	0.2

TABLE VII.—TYPICAL PROCEDURE B TITRATIONS OF EXPERIMENTAL FORMULAS

Formula		44	45	46	47	48	49	50	51	52	53
		Liquids		Powders					Tablets		Powder
Aluminum hydroxide gel (Gm.)		0.625	0.625	..	..	..	..	..	...	...	..
Aluminum hydroxide gel dried powd. "A"		...	...	..	1.0	0.5	0.33	..	...	0.25	0.25
Aluminum hydroxide gel dried powd. "B"		...	...	1.0	..	..	..	..	...	...	..
Bone phosphates		...	...	..	..	0.5	0.67	1.0	...	...	..
Glycine		...	...	..	..	..	..	..	..	0.25	0.25
Aluminum dihydroxyglycinate		...	...	..	..	..	..	..	0.5	...	..
Peanut oil		...	2.8	..	..	..	..	..	...	...	..
Methylcellulose		...	...	..	..	..	..	..	...	0.025	..
Minutes	Total ml. 0.1 *N* HCl (Equivalent) Added										
0	50	1.07	1.00	1.02	0.96	0.96	0.88	1.00	1.00	0.88	0.94
1	50	1.87	1.65	2.49	1.05	3.40	2.73	3.56	2.42	1.08	1.30
3	50	3.32	3.08	3.42	1.11	3.45	2.83	3.66	3.13	1.20	1.33
5	50	3.68	3.53	3.71	1.38	3.52	2.82	3.68	3.38	1.29	1.39
10	50	3.76	3.68	3.87	3.40	3.5	2.90	3.69	3.58	1.50	1.50
20	70	3.67	3.65	3.60	3.42	2.62	2.34	3.52	3.33	1.34	1.42
30	90	3.63	3.56	3.64	3.33	2.33	2.29	3.37	2.82	1.18	1.29
40	110	3.56	3.50	3.58	3.24	2.23	2.32	2.90	2.20	1.00	1.08
50	130	3.49	3.38	3.53	3.20	2.19	2.35	2.30	1.74	...	1.00
60	150	3.39	3.20	3.51	3.13	2.13	2.31	1.78	1.34	...	..
70	170	3.12	2.31	3.47	3.08	1.92	2.21	1.42	1.12	...	..
80	190	2.47	1.65	3.40	2.98	1.65	2.06	..	...	...	..
90	210	1.72	1.34	3.36	2.74	1.46	1.77	..	...	...	..
100	230	...	...	3.30	2.20	..	1.50	..	...	...	..
110	250	...	...	3.19	1.38	..	..	..	...	...	..
120	270	...	...	2.97	..	..	..	..	...	...	..
130	290	...	...	2.18	..	..	..	..	...	...	..
140	310	...	...	1.65	..	..	..	..	...	...	..
150	330	...	...	1.36	..	..	..	..	...	...	..
Maximum *p*H attained		3.8	3.7	3.9	3.4	3.6	2.9	3.7	3.6	1.5	1.5
Minutes to Reach *p*H 3.5		5	5	5	∞	5	∞	1	10	∞	∞
Hours above *p*H 4		0	0	0	0	0	0	0	0	0	0
Hours above *p*H 3		1.2	1.0	2.0	1.3	0	0	0.5	0.3	0	0
Hours above *p*H 2		1.3	1.2	2.2	1.7	1.0	1.3	0.8	0.7	0	0

duplicate this performance at *p*H 4–6.8 with a 5-ml. dose. Fluid product 23 is effective in this range at a 10-ml. dose.

Tablet product 35 (Table V) gave the most prolonged action at *p*H 3 to 4 holding the *p*H above 3 for 1.3 hours with one tablet and for two hours with two tablets.

Liquid product 9 (Table IV) was similarly effective in this range at a dose of 10 ml.

Antacid Chemicals.—Although certain ingredients in antacid formulas serve only as pepsin or other enzyme inhibitors, demulcents, protectives, or the like, a study of their buffer activity was made to assist in predicting the buffer capacity of a compound antacid.

Our experiments using a gram or more of ingredient indicate that bentonite, methylcellulose, silica gel, Veegum®, kaolin, aluminum silicate, guar gum, and sodium cellulose sulfate have no buffer capacity. Sodium carboxymethylcellulose, bismuth subcarbonate, and calcium gluconate are negligible (Tables II and VI). Calcium glycero-

phosphate is a very weak buffer at *p*H 2 or less. Bone phosphates, glycine, calcium citrate, and dicalcium phosphate are potentially useful antacids at *p*H 2–3 (Tables II, VI, and VII). Aluminum hydroxide and magnesium trisilicate (especially in in combination), or colloidal magnesium silicate are useful below *p*H 4 while calcium carbonate is useful at *p*H 5 to 6, sodium bicarbonate and magnesium carbonate at *p*H 7 to 8, and magnesium oxide at *p*H 9 to 10.

Experimental Antacids.—Experimental antacid formulas are given in Tables III and VII. Not all of the antacids which appeared promising with Procedure (A) studies were tested by Procedure (B) since many of these compositions fermented or were otherwise unstable or unpleasant. Liquid products containing glycine are a good example since these darkened or grew yeasts and molds.

Several experiments were conducted to determine the effect of adding oils to antacid formulations. Titrations 44 and 45 (Table VII) indicate that the addition of peanut oil 28% to aluminum hydroxide gel only slightly reduced the antacid activity. Comparison of commercial fluids 2 and 3 (Table I) show that similar suspensions containing 10% mineral oil are about equally active.

It was observed that fluids containing equal parts of glycine and aluminum hydroxide gel gave neutralization values almost exactly those of aluminum dihydroxyglycinate (Table III, Examples 14–16). It is probable that the ingredients which were in about the right proportions may actually have formed this compound in the presence of water. Dry powder mixtures of glycine and dried aluminum hydroxide gel in those proportions (Table VII, Examples 51–53) do not show this effect. Examples 52 and 53 (Table VII) also show the slight depressant effect produced by methylcellulose in antacid formulas.

Titrations 47–50 (Table VII) demonstrate the difficulties in predicting *p*H values of mixtures when the titration curves of the ingredients are known. Here a 1:1 mixture of bone phosphates[1]-dried aluminum hydroxide gel gave values intermediate between the two early in the titration, but quickly dropped to values resembling those of aluminum phosphate. Mixtures at a ratio equivalent to aluminum phosphate (2:1) gave a maximum *p*H value of 2.9 although the parent ingredients had maxima of 3.7 and 3.4, respectively. This 2:1 mixture thus closely resembles the buffer curve of aluminum phosphate. Such experiments show that actual titrations [such as Procedure (B)] are needed to characterize a compound antacid.

Titrations 46 and 47 (Table VII) illustrate the wide variation in two lots of dried aluminum hydroxide gel U. S. P. supplied by the same manufacturer. The ml. of acid neutralized to *p*H 2 by one gram of powder vary here from 230 to 290 ml. of 0.1 *N* HCl. The corresponding theoretical length of action varies from one hundred to one hundred thirty minutes. This bears out the variations noted with Procedure (A) on four lots of U. S. P. dried aluminum hydroxide gel and three lots of U. S. P. magnesium trisilicate (Table II). This further supports the desirability of additional U. S. P. tests to standardize official antacids as recently proposed by Gore, *et al* (9). We are currently investigating published procedures and modifications of these in an effort to establish a simplified standard method.

[1] Essentially tricalcium phosphate.

SUMMARY

1. The *in vitro* antacid activities of twenty-four commercial products, twenty-four antacid ingredients, and eighteen investigational formulas have been studied.

2. The disagreement in the literature as to the effective *p*H range of antacids is noted. The results show the effectiveness of various formulas at these *p*H ranges.

3. Combinations of aluminum hydroxide and magnesium trisilicate appear superior in the range of *p*H 4 or below. The mixture of calcium carbonate, magnesium trisilicate, and magnesium carbonate (12:2:1) stands out at *p*H 6.8 or below. In the Sippy treatment range of *p*H 7 and above, magnesium oxide-sodium bicarbonate (1:1) is the most effective.

4. Comparisons of buffer capacities of antacid ingredients show (1) no activity (bentonite, kaolin, aluminum silicate, silica gel, Veegum®, sodium cellulose sulfate, methylcellulose, guar gum), (2) very slight activity (sodium carboxymethylcellulose, bismuth subcarbonate, calcium gluconate, calcium glycerophosphate), (3) effective activity (aluminum phosphate, bone phosphates, glycine, polyamine methylene resin, calcium citrate, dicalcium phosphate, aluminum hydroxide, magnesium trisilicate, colloidal magnesium silicate, calcium carbonate, sodium bicarbonate, magnesium carbonate, magnesium oxide).

5. Clinical coefficients are proposed as a means of converting *in vitro* data into useful *in vivo* data.

6. Interactions among antacid ingredients sometimes produced new buffer compounds making predictions of *p*H difficult. As a result actual titrations are required for new combinations.

7. Variations in official grade antacid materials suggest the need for further test procedures.

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Iodine as a Virucidal Agent*

By LOUIS GERSHENFELD†

The effectiveness of iodine is detailed in the field of therapy and especially in the fields of prophylaxis and sanitation of many infections caused by viruses. The use of a suitable antiseptic for preparing the surface to be traumatized, either by injections or by operative procedure, is of great importance. Of the local antiseptics tested, iodine tincture was found to be most effective in quickly destroying the poliomyelitis virus. In the author's laboratory, concentrations of free iodine as may be used in preparing a mouth wash were found to be capable of destroying polio virus.

THE TERM "VIRUS" is derived from the "Latin—a slimy or poisonous liquid, thus also poison or venom; from Sanskrit, 'visham,' a poison—thus, virulent. The term, in the sense of poison, has been used in English since 1500. The modern idea of a virus as an infecting agent too small to be seen with the microscope was introduced in 1889 when Beijerinck suggested that a 'virus' was the cause of mosaic disease in plants. Later Loeffler demonstrated that foot-and-mouth disease was due to a filterable virus. Recent studies with the electron microscope indicate the more precise nature of viruses" (1).

In "Bergey's Manual of Determinative Bacteriology" (2), viruses are grouped under the order *Virales*. The following brief description is given: "*Viruses.*—Etiological agents of disease, typically of small size and capable of passing filters that retain bacteria, increasing only in the presence of living cells, giving rise to new strains by mutation, not arising *de novo*. A considerable number of viruses have not been proved filterable; it is nevertheless customary to include these viruses with those known to be filterable, because of similarities in other attributes and in the diseases induced. Some not known to be filterable are inoculable only by special techniques, as by grafting or by use of insect vectors, and suitable methods for testing their filterabiiity have not been developed; moreover, it is not certain that so simple a criterion as size measured in terms of filterability will prove to be an adequate indicator of the limits of the natural group. Cause diseases of bacteria, plants and animals."

In the Act of Congress which controls the production of biological products for interstate sale within the jurisdiction of the United States and which was approved July 1, 1902, and re-enacted as part of the Public Health Service Act (Public Law 410—78th Congress) approved July 1, 1944 (3), we find among regulations issued a definition of a virus as follows: "A virus is a product containing the minute living cause of an infectious disease." A more vague designation is the term "an analogous product," defined as follows: "A product is analogous to a virus if prepared from or with a virus or agent actually or potentially infectious, without regard to the degree of virulence or toxicogenicity of the specific strain used."

"Viruses are infectious agents." "Viruses are infectious agents which, in size, adjoin and extend downward from the bacteria and protozoa" (4). These definitions noted most frequently in textbooks recognize viruses only on the basis of their ability to produce infection in susceptible cells or hosts. Sole dependence on detectable abnormality in a host or pathogenicity as an index of viral activity or the existence of a virus is a concept difficult to accept. A detailed consideration of virus classification and nomenclature including many aspects of specific problems concerning certain virus groups was presented at a recent conference (5).

Most investigators regard viruses as microorganisms, and that essentially they do not differ

* Received August 27, 1954, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
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fundamentally from other living microscopic organisms. They claim that the variations seen among viruses morphologically, biologically, and in other respects are characteristic of all living microscopic forms. In contrast to this opinion that viruses are self-determinative and microscopic organisms in nature, there is the concept that they are nucleoprotein molecules, though there has been no presentation of the clinical evidence of the structure of such molecules. Such a concept carries a possible implication that viruses are inanimate and perhaps are produced by host cells. It also has been noted "that viruses alter the metabolism of the host cells to favor the production of new virus particles to the detriment of the host cell" (6). It is possible that the elucidation of these queries by the vast amount of research now in progress will bring about in the not too distant future more definite data concerning the origin and reactions of viruses.

Perhaps the only features uniting the virus group by tacit definition are that they are smaller than organisms visible under ordinary microscopes and that they have not been cultivated *in vitro*. Certainly no one claims a uniformity of structure, origin, or method of multiplication. They constitute a heterogeneous group of agents which operate in a manner similar to that of bacteria and protozoa, except that when infective, they require susceptible host cells for activity and multiplication. The primitive state of our knowledge concerning the biology and biochemistry of viruses is known to all. Data concerning even the basic problems of viruses are not as yet available. Viruses have defied definition as a distinct biological class. A rigid definition is not possible unless we accept Luria's broad definition of a virus as an "exogenous submicroscopic unit capable of multiplication only inside specific living cells" (7). Viruses then are obligatory intracellular parasites and appear to be formed of nucleoproteins.

In view of what has been said, it is explainable why some workers are of the opinion that "viruses defy precise definition." They are known today for what they do rather than what they are. It is for this reason that the pathogenic agents grouped under viruses are known best for their many characteristics and perhaps, for the present, justify the unique position held by viruses. Certain diseases caused by viruses have been known clinically for centuries, though the existence and the nature of the agents causing them have been recognized only recently. Some of the virus diseases rival in importance other diseases of man, lower animals, and plants. The following are the more important virus diseases most frequently encountered in man: Chickenpox (varicella); Common Cold; Dengue (breakbone) Fever; Encephalitis; Herpes Labialis (cold sore); when the mucosa of the genital tract is attacked, the virus may be designated as Herpes Genitalis; when accompanied by fever, the virus may be designated as Herpes Febrilis; Herpes Zoster (shingles); Infectious (epidemic) Hepatitis (catarrhal jaundice); Influenza (epidemic); Lymphocytic Choriomeningitis (acute aseptic meningitis); Lymphogranuloma Venereum (venereal bubo); Measles; Molluscum Contagiosum; Mumps (epidemic parotitis); Pappataci (sandfly or three day) Fever; Phlebotomus Fever; Poliomyelitis (epidemic infantile paralysis); Psittacosis (ornithosis); Rabies (hydrophobia); Rubella (German measles); Smallpox (variola); Trachoma (granular conjunctivitis); Virus Pneumonia (primary atypical pneumonia); Warts; and Yellow Fever.

In addition to these virus diseases of man, the following more important virus diseases of plants and lower animals are included: African Horse Sickness; Canine Distemper; Foot-and-Mouth Disease in Cattle, Sheep, and Goats; Fowl Pest; Fowl Plague; Fowl Pox; Fowl Sarcoma; Newcastle Disease of Chickens; Rinder Pest; Silkworm Jaundice; and Tobacco Mosaic Disease.

VIRUS INACTIVATION BY CHEMICALS

A "virucide" as the term is generally used refers to an agent which destroys or inactivates viruses. The agent may be physical (such as elevated temperatures and ultraviolet light of suitable wave length), biological (such as antibodies and other components of the host's defense mechanisms), or chemical. It is the chemical virucidal agents and specifically the effect of iodine on different viruses with which this presentation is concerned. Virus inactivation or destruction by chemicals, as in the case of microorganisms in general, varies depending upon the chemical agent and the many conditions of examination. In the case of viruses especially, protection is afforded by the presence of tissue cells, their constituents and products produced, and proteins in general. There also exists here the overall problem of noting whether the action exerted is directly on the virus, or primarily on the host cell or host tissue, or on tissue metabolites, or perhaps on an agent produced by host-virus interaction.

There are few new data that one can offer in the field of therapy of most infections caused by viruses. Since within recent years many chemicals have been found effective against the agents causing bacterial and even protozoan diseases, it has become of great importance to know more concerning chemicals and drugs which might be effective against viruses. New advances will undoubtedly be forthcoming within the next decade. It must be recognized

that the many studies dealing with the effectiveness of chemical agents are varied, and frequently apply to methods, which are not necessarily standard procedures, or at times may not even be comparable to conditions as found in practice nor simulate those which are present in nature. There is also the problem of the selective action of a number of different antiseptics on the same viruses and of the same antiseptic on different viruses. There is, however, something new which is encouraging, as it concerns both the pathogenic virus (poliomyelitis) and iodine in the field of prophylaxis and sanitation.

Poliomyelitis Virus.—Among the diseases caused by viruses, considerable interest has developed within recent years concerning all aspects of the virus causing poliomyelitis and suitable antiseptics and disinfectants which are effective in its inactivation.

Faber of the Stanford University School of Medicine in a five-page editorial on postinoculation poliomyelitis (8) indicates the following: "poliomyelitis following inoculations of immunizing materials of various sorts, with paralysis in the inoculated limb may be due to accidental introduction into peripheral nerves of virus from the patient's own skin (perhaps the more likely explanation), or from previously contaminated needles or syringes inadequately sterilized, rather than to exacerbation and localization of pre-existent infection. . . . The following practical suggestions emerge from the foregoing discussion as of potential preventive value: 1. Syringes and needles used for injections of all sorts should be sterilized by autoclaving; or, if this is impractical, by boiling for twenty minutes. 2. A strong antiseptic should be used, preparatory to injections, both on the patient's skin and on the stopper of the vial. Alcohol or soap and water cannot be regarded as safe. Until more information is available as to the choice of antiseptic 2% tincture of iodine is suggested."

Banks (9) points to "another new factor, which may have had the effect in recent years of converting subclinical poliomyelitis in children's wards into the paralytic form—namely, the frequency with which intramuscular penicillin injections in concentrated doses are given to children in hospitals." Faber, *et al.* (10), in an experimental study noted that "1. Application of poliomyelitis virus to the pharynx of cynamolgus monkeys, followed by enucleation of the tonsils, was regularly followed after seven to ten days by primary bulbar paralysis, accompanied less often by high spinal paralysis. . . . 2. Intrathalamic inoculation of virus followed three days later by tonsillectomy was regularly followed within two more days by bulbospinal paralysis in which all levels of the cord were involved. . . . 3. After oropharyngeal swabbing with virus and no tonsillectomy, spinal paralysis usually resulted. . . . 4. With pharyngeal application of virus followed by 2% tincture of iodine and then by tonsillectomy all six animals remained well."

Hayes (11) in presenting a survey of poliomyelitis indicates "that recent tonsillectomy and adenoidectomy predispose to poliomyelitis, especially the bulbar type." He indicates that "Experimentally, the occurrence of poliomyelitis following the enucleation of tonsils was prevented by applying 2% tincture of iodine over the inoculated area of the pharynx."

Faber and Dong (12, 13) in a recent presentation on poliocidal activity of antiseptics summarize as follows: "Evidence herein presented that poliomyelitis virus may, in rare instances, be introduced into the body by penetrating local trauma (tonsillectomy, injections) emphasizes the importance not only of proper sterilization of instruments, needles, and syringes but also of preparing the surface to be traumatized with an antiseptic capable of quickly destroying poliomyelitis virus, preferably within half a minute. Of the local antiseptics tested, mild (2%) tincture of iodine was found to be highly effective; tincture of merthiolate moderately so; tincture of metaphen and 2% hexachlorophene only slightly effective; and ethyl alcohol (70%) and tincture of zephiran, ineffective, all within the stated time limit. The mild tincture of iodine is therefore recommended for routine use. Antiseptic should also be applied to the stopper of the vial before withdrawing material for injection. While the preventive prophylactic value of local antisepsis against poliomyelitis is undoubtedly limited, its routine use is clearly indicated and might prevent some occurrences of localized paralysis." Faber (13) in reply to a question concerning a quaternary ammonium compound answered, "I feel that 2% (not 7%) iodine is the best of the local antiseptics because of its broad spectrum, being effective against both bacteria and at least the small viruses, whereas benzalkonium has value, so far as I know, only against bacteria. Certainly it is completely ineffective against poliomyelitis virus."

Heinertz and Vahlne (14) of the State Institute of Public Health in Stockholm in a consideration of poliomyelitis as related to public water supply "conclude that poliomyelitis infections may be water-borne in places where the sources of drinking water are not free from fecal pollution. Chlorination is deemed insufficient to eliminate poliomyelitis virus." Chang and Morris (15) in a report of practical studies of water disinfection found that inactivation of poliomyelitis virus "has been observed with iodine residuals of a few parts per million and contact times of five to ten minutes."

In an experimental study at the author's laboratory (16) the following was noted: (*a*) A concentration of 0.375 mg. free iodine per ml. prepared from Heliogen® tablets,[1] prevented within one minute the appearance of cytopathogenic effects in monkey kidney cells due to Type I (Mahoney strain) and Type II (MEF 1 strain) poliomyelitis virus. The viruses were used in a concentration of 10^{-4}. (*b*) Against Type III (Saukett strain) poliomyelitis virus 10^{-3}, only 0.125 mg. free iodine per ml., prepared from Heliogen® tablets, was needed to prevent within one minute the cytopathogenic effects of the virus in the monkey kidney cells.

Influenza Virus.—Dunham and MacNeal (17) prepared mixtures of antiseptics and influenza virus and allowed "them to remain in contact for three minutes, diluting the mixtures to the point where they would not be toxic for chick embryos and then injecting the material into embryonated eggs." They noted that "Tincture of Iodine, U. S. P. XII, was effective when diluted to 0.1% strength. This

[1] Marketed by Heliogen Products, Inc., Long Island City 3, N. Y.

solution contained iodine in a concentration of 0.07 mg. per ml. When the official tincture was diluted to 0.01% strength, it was without effect. Lugol's solution, U. S. P. XII, diluted 1:100 inactivated the virus, whereas it failed to do so when it was diluted 1:1000." Knight and Stanley (18) of the Rockefeller Institute noted "the effect of 20 different chemicals on the activity of purified preparations of the PR8 strain of influenza virus obtained by differential centrifugation." They found by tests in chick embryos and in mice that iodine "destroyed virus infectivity promptly, even in 0.0005 *N* solution. Iodoacetamide, which may or may not act as a specific reactant for protein SH groups, was found to inactivate the virus at once in 0.5 *N* solution and more slowly in 0.05 *N* solution."

Stone and Burnet (19) in an experimental ***in vitro*** and ***in vivo*** study on influenza virus "found that iodine vapour is a very potent inactivating agent." They noted that "Minute concentrations of iodine vapour of the order of 0.1 p. p. m. by volume have been found capable of destroying the infectivity of a mist of influenza virus droplets." Of interest is their comment, "Perhaps the most striking of the early experiments was the demonstration that mice could be protected completely merely by painting a little tincture of iodine on their snouts several minutes before they were placed in the bin and exposed for ten minutes to a concentration of mist capable of producing heavy infection or death in control mice." Burnet, Holden, and Stone (20) in reporting on the action of iodine vapour on influenza virus in the form of a mist, produced to simulate the process of natural droplet infection, indicate "that it may be worth considering iodine vapour as a practical means of limiting indoor infection during an influenza epidemic." Wagner (21) noted that an iodine compound was effective. He states: "In saline solutions of $NaIO_4$, influenza A virus rapidly loses infectivity for chick embryos." He indicates that it is an effective inactivator "of the PR8 strain of influenza virus by virtue of a nonspecific oxidation."

It is known from experimental studies that some viruses are adsorbed on red blood cells and certain other cells and are subsequently eluted spontaneously. Heating some of these viruses (at 56° for an hour) does not prevent them from being adsorbed by the red blood cells, but the capacity for self-elution is lost. Furthermore, it was found that treating the virus with periodate had an effect similar to that of heating the former, in that the virus retained its properties of adsorption on red blood cells but the capacity for self-elution was lost. De St. Groth and Graham (22) reported that by treatment with periodate, "the infectivity, enzyme activity, antigenicity, and haemagglutinin of influenza viruses can be destroyed." They present findings indicating that "metaperiodate modifies virus receptors." Hirst (23) found that "Influenza virus, treated with sodium periodate, was adsorbed well on red cells but lacked the capacity for spontaneous elution." The same worker (24) noted that the influenza virus receptors of fowl red cells and the influenza virus inhibitor of normal rabbit serum "are readily destroyed by sodium periodate."

In the same type of investigation as above, Chu and Morgan (25) reported that not only influenza virus but also mumps virus were not eluted after periodate treatment. Wagner and Stacy (26) found that *in vitro* sodium periodate inactivated influenza A virus up to 50,000 infective doses.

Vaccinia Virus.—Kaiser (27) aerated vaccinia virus (suspended in water) with air, which had first been passed through a 10% alcoholic solution of iodine. The virus was inactivated within thirty seconds and could not be reactivated. By suspending the virus directly in dilute iodine solutions, the minimum concentration of iodine for inactivation was found to be 1:1,000,000. Similar results were obtained with myxoma virus. "Tests with vaccinia virus and others with purified influenza virus suggest that inactivation occurs readily with oxidizing agents (potassium permanganate, iodine), while foot-and-mouth-disease virus is resistant" (28). Dunham and MacNeal (29) prepared mixtures of vaccinia virus and antiseptics, allowed them to remain in contact for three minutes, diluted the mixtures to a point where they would not be toxic for chick embryos, and then injected the materials into embryonated eggs. Under these conditions, the iodine tincture then official (containing 7% iodine) "was effective when diluted to 1% strength. . . . When the official tincture was diluted to 0.1% strength, it was without effect." Of interest are the following comments concerning the preparation of the human skin before vaccination. Shaw (30) states: "I think it might be good practice to use iodine for skin preparation before vaccination. . . . Even though many may insist that this will interfere with successful vaccination, it won't do so at all." Faber (31) comments on vaccinia as follows: "I have also found that preparation of the skin with 2% iodine does not interfere with takes." In view of the experimental *in vitro* findings mentioned above and the comments by Shaw and Faber, it is possible that in the multiple puncture method and proper technique used in smallpox immunization, the iodine may be washed away or sufficient iodine may not always be carried along with the vaccine into the subcutaneous tissues, to exert an inactivating effect on the attenuated smallpox virus used in the immunization technique in humans.

Herpes.—Herpes Simplex, an acute inflammatory affection, is characterized by the formation of pinhead to small pea-sized, grouped vesicles, occurring about the face (Herpes Facialis) or genitalia (Herpes Progenitalis). If found about the lips, it is usually known as the ordinary fever blister or the cold sore. Hruszek (32) reported that the application of tincture of iodine before the application of herpes virus prevented the development of the infection. On the other hand, the application of this antiseptic after inoculation of the scarified area did not interfere with the growth of the virus. Thygeson and Hogan (33) treated herpes of the cornea with different drugs and reported iodizing the cornea was very successful.

Herpes Zoster, an acute inflammatory disease, is characterized by the appearance of several groups of vesicles on slightly elevated and inflamed areas of unilateral distribution and corresponding to the cutaneous course of a nerve trunk. Zoster in any region is commonly known as "shingles." The causative factor is a virus. Ruggles (34) mentioned the specific effects of an iodine compound, sodium iodide. He indicated that the effects

were not germicidal, but it was "supposed to cause lysis and release bacteria and promote resolution of diseased tissues. However, in some conditions, especially when administered intravenously, they appear to have a direct action." Phillips and Morginson (35) state "Under our direct observation and on our recommendations, we have seen sodium iodide give almost phenomenal relief in 11 cases of Herpes Zoster and one case of Herpes Simplex." Other workers have also reported on the success of this treatment (36, 37). Binder and Stubbs (38) indicate that: "Roentgen therapy in filtered doses to the posterior nerve root affected, in combination with the intravenous use of sodium iodide, is occasionally successful."

Rabies.—Shaughnessy and Zichis (39) in a study on the prevention of experimental rabies "in which treatment of wounds contaminated with rabies virus was instituted within thirty minutes" concluded: "In tests using a limited number of guinea pigs, the results of applying tincture of iodine within thirty minutes compared favorably with the results obtained following treatment with either fuming nitric acid or soap solution."

Common Colds.—Various workers during the past several decades have reported the beneficial effects of iodine as an abortive for the common cold, for use in vasomotor rhinitis, and for relieving the coryza of allergy and upper respiratory infections. There are no data available to indicate whether iodine has or has not a specific action against the virus of the common cold. Its action is generally regarded as nonspecific. Inasmuch as many workers expressing as does Kovnat (40) a "belief that it has been successful in aborting the common cold in a great majority of cases," herein are listed a few presentations (41–43).

Trachoma.—The first decade of the present century lists many reports on the treatment of trachoma (granular conjunctivitis) and its complications with iodic acid, iodates, and other iodine compounds. Later Neumann (44) and Sharma (45) and others reported on the successful use of iodine in the treatment of trachoma. The latter worker makes the following interesting comment: "what has struck me most in the treatment of Trachomatous complications in Trachoma, with iodine, is its cheapness and being easily available in every dispensary."

Tobacco Mosaic Virus.—Anson and Stanley of the Rockefeller Institute (46) indicate that "tobacco mosaic virus is an SH protein of the egg albumin type." They note that "Iodine is the only reagent known to abolish the SH groups of egg albumin and tobacco mosaic virus by reaction with the native form of these proteins." They summarize as follows: "The SH groups of tobacco mosaic virus or their precursors can be abolished by reaction of the native form of the virus with iodine. Tobacco mosaic virus whose SH groups have been oxidized beyond the S-S stage by iodine but whose tyrosine groups have not been converted into di-iodotyrosine groups still retains its normal biological activity as shown by the number of lesions it causes on *Nicotiana glutinosa* plants and by the characteristic disease produced in Turkish tobacco plant. . . . If enough iodine is added to tobacco mosaic virus or if the iodine reaction is carried out at a sufficiently high temperature, then the tyrosine groups are converted into di-iodotyrosine groups and the virus is inactivated. Tobacco mosaic virus can be almost completely inactivated by iodoacetamide under conditions under which iodoacetamide reacts with few if any of the protein's SH groups." Agatov (47) of the Moscow Pedagogical Institute gave special attention to the effect of iodine on the activity of tobacco mosaic virus and in a series of studies noted "it may be seen that with a pH equal to 5.5–6.0, iodine had either no influence at all, or had but a slight effect upon the activity of the virus in question. When the medium had a more acid or a more alkaline reaction, iodine caused a considerable reduction in the activity of the tobacco mosaic virus, which attained 100% with a pH 4.5 and 8.0."

Other Virus Diseases.—Cohen (48) reports on "six cases of verrucca plana juvenilis cured by the intramuscular injection of bismuth and the local application of tinctura iodidorum." Warts on cattle are discussed by Creech, *et al.* (49). They indicate that: "Before warts become excessively large they may be destroyed by daily applications of glacial acetic acid or tincture of iodine."

Cunningham (50) in studies on "the effect of certain chemical agents on the virus of Newcastle disease (pneumoencephalitis) of chickens" found that "tincture of iodine, 2.5% and 1%" was effective and "completely inactivated the virus during the three-minute reaction period" in the technique used. McCulloch (51) reported "that tincture of iodine diluted 1:400 inactivated the virus of fowl pox while 1:800 failed."

Goebel, *et al.* (52), studied "the action of potassium and lithium periodate on the virus of Western equine encephalomyelitis." They found that "the pathogenic action of the virus was destroyed" and its specific antigenicity was lost. Radeleff (53) noted that "Sodium iodide, on the basis of results obtained in the 43 cases (treated), proved to be a valuable agent in the therapy of infectious equine encephalomyelitis."

Cunningham and Stuart (54) reported that the virus of infectious bronchitis of chickens was inactivated within three minutes by 1% Lugol's Solution.

SUMMARY

1. Derivation, definition, and important characteristics of viruses as well as some features uniting the virus group are presented. The more important virus diseases most frequently encountered in man, in lower animals, and in plants are mentioned. Chemical virucidal agents and specifically the effect of iodine on different viruses (*in vitro* and *in vivo*) are considered. More detailed information is presented especially concerning the effective inactivation by iodine and certain iodine compounds of the viruses of poliomyelitis, influenza, herpes, vaccinia, rabies, tobacco mosaic, and others.

2. In recent years, evidence has been presented that poliomyelitis virus may be accidentally introduced into the body from the patient's skin or mucous membrane by penetrating local trauma (as after tonsillectomy and adenoidec-

tomy) or following inoculations of immunization materials or injections of various substances. This may be prevented by the prior application of solutions of free iodine of suitable concentration. Under certain conditions, poliomyelitis infections may be water-borne and here too, inactivation of the virus has been noted when using solutions of free iodine.

3. In the author's laboratory, in an experimental study, Type I (Mahoney strain), Type II (MEF-1), and Type III (Saukett Strain), Poliomyelitis virus were inactivated within one minute by weak iodine solutions of strengths as are employed in preparing a mouth wash. The iodine solutions were prepared freshly from Heliogen® tablets.

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The Blood Level Determinations of PAS and Isoniazid with Vanillin*

By EDWARD N. DEEB† and GUY R. VITAGLIANO‡

A relatively simple spectrophotometric method is described for the determination of PAS and isoniazid blood levels. The method is based upon the formation of soluble yellow compounds resulting from the addition of vanillin solution to a solution of PAS or isoniazid at a controlled *p*H. Recoveries of known quantities added to plasma demonstrate that the method is accurate to ±0.9 per cent for PAS and ±0.4 per cent for isoniazid.

SEVERAL METHODS for the assay of PAS (5, 6, 8) and isoniazid (1, 3, 7) have appeared in the literature. Due to the extremely low concentrations of these drugs normally encountered *in vivo*, only a few of the assay procedures are applicable to blood-level determinations. However, two (6, 8) of these involve spectrophotometric methods in which color development duplicated is extremely weak in the range encountered.

* Received August 27, 1954, from the Veterans Administration Hospital, Rutland Heights, Mass. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.
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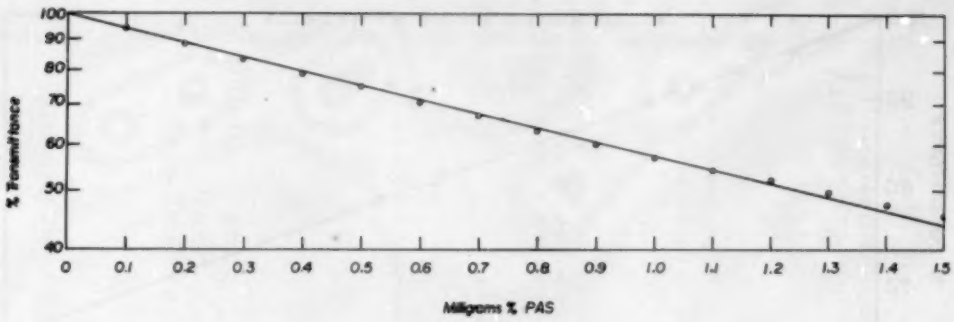
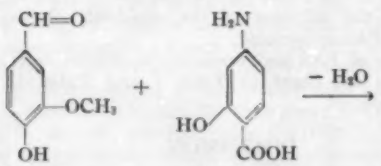


Figure 1.

Considerable difficulty was experienced with the use of a procedure for isoniazid published by Jacobs (1). Development of clear protein-free blood filtrates was unpredictable due to insolubility of excess tungstic acid produced in the highly acid media. And again, color development in the low range expected, usually below 3 μg. per ml., is barely discernible spectrophotometrically.

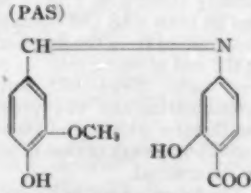
Published methods cited above for assaying low concentrations of PAS and isoniazid consistently proved unsatisfactory for blood level studies. The study presented was undertaken in response to several requests for blood-level assays on patients undergoing PAS and isoniazid therapy. The method developed is an adaptation of a procedure previously reported for the determination of PAS and isoniazid in pharmaceutical preparations (2).

Chemistry of the Reactions (4).—1. PAS reaction with the formation of an azomethine:



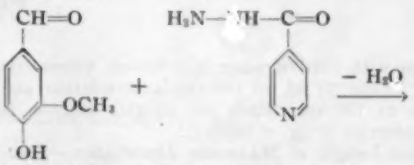
3-Methoxy-4-hydroxybenzaldehyde (vanillin)

p-Aminosalicylic acid (PAS)

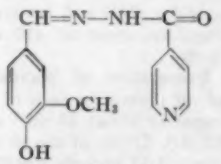


N-(3-Methoxy-4-hydroxybenzylidene)-p-aminosalicylic acid (yellow compound)

2. Isoniazid reaction with the formation of an aldimine:



(Vanillin) Isonicotinic acid hydrazide



1-Isonicotinyl-2-(3-methoxy-4-hydroxybenzylidene)-hydrazine (yellow compound)

EXPERIMENTAL

Reagents.—Vanillin 10% solution is prepared by dissolving 10 Gm. of vanillin, U. S. P., in 50 ml. of 95% ethyl alcohol and then diluting to 100 ml. with distilled water. Vanillin 2% solution is made by dissolving 2 Gm. of vanillin, U. S. P., in 25 ml. of 95% ethyl alcohol and then making up to volume with distilled water in a 100-ml. flask.

Trichloracetic acid 20% aqueous solution, sulfuric acid 5 *N* and 1 *N* are prepared in accordance with recognized standard methods using reagent grade chemicals.

Para-aminosalicylic Acid 10 mg. % Standard.—Weigh accurately 138 mg. of sodium *p*-aminosalicylate[1] (equivalent to 100 mg. of PAS). Dissolve in distilled water, diluting to 100 ml. Take 10 ml. of this 100 mg. % solution and dilute to 100 ml. This results in a 10 mg. % standard solution.

Isoniazid 20 μg. per ml. Standard.—Weigh accurately 100 mg. of pure isoniazid[2] and make up to

[1] Supplied by Merck & Co.
[2] Supplied by Hoffman-LaRoche as pure Rimifon #50-2.

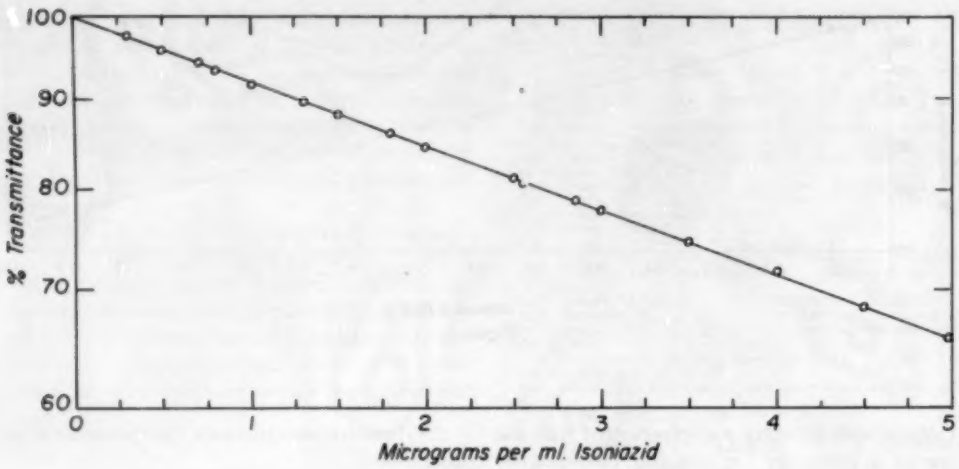


Figure 2.

volume with distilled water in a 500-ml. volumetric flask. Take 10 ml. of the resultant solution and dilute to 100 ml. Each ml. of this solution is equivalent to 20 μg. of isoniazid.

Wave Length of Maximum Absorbancy.—Spectral transmittance curves were plotted using several concentrations of the yellow reaction products of both PAS and isoniazid. The maximum absorbancy for PAS and isoniazid reaction products was found to occur at 410 mμ and 400 mμ, respectively.

Preparation of Standard Curves.—PAS—To 2 ml. of plasma in each of eight screw-capped tubes (capacity about 25 ml.) add 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml. of the 10 mg. % PAS standard solution. Add enough distilled water to each of the eight tubes to bring volume to 12 ml. per tube. Mix by swirling, and add 4 ml. of 20% trichloracetic acid to each tube. Stopper the tubes and shake vigorously. Centrifuge at 1500 r. p. m. for five minutes and filter through a highly retentive quantitative filter paper. Place 8 ml. each of the clear protein-free filtrates in 19 x 150-mm. spectrophotometric cuvettes. Add 1 ml. of 10% vanillin and 1 ml. of 5 *N* sulfuric acid to each of the tubes. Mix well by swirling and allow to stand ten minutes. Solutions are then compared at a wave length of 410 mμ against the control which consists of 8 ml. distilled water, 1 ml. vanillin 10%, and 1 ml. 5 *N* sulfuric acid.

Figure 1 indicates the points obtained and also graphically illustrates conformity to Beer's law. Other points were later determined and included in Fig. 1. Since the final dilution represents 1 ml. of plasma in 10 ml. of dilution, the reading must necessarily be multiplied by 10 to obtain actual blood level.

Isoniazid.—To 4 ml. of plasma in each of five screw-capped tubes add 1, 2, 3, 4, and 5 ml. of the 20 μg. per ml. isoniazid standard solution. Add enough distilled water to make the volume 8 ml. per tube. Then add 8 ml. of 20% trichloracetic acid to each tube, stopper tubes, and shake vigorously. Centrifuge at 1500 r. p. m. for five minutes and filter through a highly retentive quantitative filter paper. Place 8 ml. each of the clear protein-free filtrates in 19 x 150-mm. cuvettes and add 1 ml. of 2% vanillin and 1 ml. of 1 *N* sulfuric acid to each of the tubes. Mix well by swirling and allow to stand ten minutes. The resultant solutions are then compared spectrophotometrically at 400 mμ against the control which consists of 8 ml. distilled water, 1 ml. vanillin 2%, and 1 ml. 1 *N* sulfuric acid. Since the final dilution represents 2 ml. of plasma in 10 ml. dilution, the reading must be multiplied by a factor of 5 to obtain actual blood level.

Figure 2 was constructed from the points obtained. Other points were determined later and included in Fig. 2. It is apparent from the graph that the reaction follows Beer's law.

Determination of Blood Levels.—Oxalated blood plasma specimens[3] were used in this study; however, comparable results may be obtained with the use of serum. The PAS test requires 2 ml. of plasma; the isoniazid test 4 ml. Place the indicated amount of plasma in a screw-capped tube and proceed as described in the preparation of the standard curve, omitting the addition of the standard solution of either PAS or isoniazid.

Results of PAS and isoniazid blood-level determinations are listed in Table I and Table II, respectively.

DISCUSSION

Most of the patients who were tested were concurrently on streptomycin or dihydrostreptomycin therapy along with PAS or isoniazid. One patient was on both PAS and isoniazid. In this case, PAS was omitted from the regimen for twenty-four hours at the end of which time an isoniazid determination was made. Since PAS interferes with isoniazid determination and vice versa, it is necessary that the therapy with one of these drugs be omitted for a sufficient length of time in order that the other may be determined.

The following points became apparent as the work progressed: (*a*) The Folin-Wu tungstic acid precip-

[3] Ten milligrams of potassium oxalate per tube.

TABLE I.—PAS BLOOD DETERMINATIONS

No.	Patient	Dose, Gm.	Time Sample Taken after Last Dose, Hrs.	Reading, % T	Plasma Level, mg. %
1	M. A. H.	4	1	58.5	9.5
2	A. H. C., Jr.	4	1	50.0	12.5
3	G. S.	4	1.5	56.5	10.5
4	J. K.	4[a]	1.5	61.5	8.5
5	M. G.	4[a]	1	61.0	8.8
6	A. R. M.	4[a]	1.5	98	0.35
7	E. W. H.	4[a]	1.5	98.5	0.25
8	W. T. H.	4	1.5	67	7.1
9	A. J. D.	4	1.5	65.5	7.5
10	E. N.	4	4	74	5.0
11	E. S. F.	4	4	88	2.2
12	C. B. F.	4	4	74	5.3
13	L. C.	2.5[a]	4	99	0.2
14	M. G.	4[a]	4	89	2.0
15	A. P. C.	4[a]	4	98.5	0.25
16	A. R. M.	4[a]	4	99	0.1
17	E. W. H.	4[a]	4	99.5	0.05
18	W. T. H.	4	4	83.5	3.2
19	A. J. D.	4	4	84	3.1

[a] Indicates patient on tablets.

Trace

TABLE II.—ISONIAZID BLOOD LEVELS

No.	Patient	Dose, mg.	Time Sample Taken after Last Dose, Hrs.	Reading, % T	Plasma Level, μg./ml.
1	J. J. G.	100	1	95.5	2.3
2	A. B.	100	1	95	2.5
3	R. O. B.	100	1	96.5	1.8
4	J. B.	100	1.5	96	1.9
5	T. W.	100	3	93	4.0
6	D. F. D.	100	4	97.5	1.2
7	L. W.	100	4	99.5	Trace
8	L. A.	100	4	98	0.8
9	E. F.	50	2	96.5	1.8

itating procedure was found to be unsatisfactory for plasma protein precipitation in this method and also in the method of Jacobs (1). Since tungstic acid is highly insoluble in an aqueous acid medium, it appears that any excess which remains after all protein is precipitated will slowly precipitate out of solution resulting in erroneously high readings. (*b*) Streptomycin, dihydrostreptomycin, and viomycin do not interfere with the tests. (*c*) In low concentrations (micrograms), in acid media, the isoniazid-vanillin reaction follows Beer's law. When a curve is plotted to follow the course of the reaction at higher concentrations (milligrams), a straight line curve is not produced (2). At these higher concentrations the reaction does not conform to Beer's law. (*d*) In an acid medium, the PAS-vanillin reaction becomes so highly sensitive that even a 0.1 mg. % solution can be easily read spectrophotometrically. (*e*) In evaluating the efficacy of PAS enteric-coated tablets, actual clinical trial is essential. This includes blood-level and 24-hour urine concentration determinations.

SUMMARY

A spectrophotometric method for ascertaining blood levels of PAS and isoniazid is presented accompanied by actual blood-level determinations demonstrating the clinical operability and ease of performance of the method. This work has been done to fulfill a need for a simple method for determining the blood concentration of the above-mentioned chemotherapeutic agents.

Although this method has been shown to be applicable for the determination of PAS and isoniazid in urine, further investigation is necessary to complete this phase of the work.

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Composition of Gum Turpentines of Pines. XXIII*

A Report on Three Mediterranean Species: *Pinus pinea* (Cultivated in California), *P. halepensis* (from Israel) and *P. brutia* (from Cyprus)

By N. T. MIROV and P. M. ILOFF, Jr.†

Gum turpentines of three Mediterranean pines have been analyzed. Of interest is the finding that a change of environment (from Italy to California) did not affect the composition of *Pinus pinea* turpentine. *P. halepensis* and *P. brutia,* which are thought by some to be synonymous, differ so much in composition of their turpentines that the two should be considered as separate species.

PINUS PINEA

Pinus pinea L. is native to the Mediterranean region, from Portugal to Syria. Its northern limits are in southern France and northern Italy. *P. pinea* has been widely planted for centuries, and it is difficult to say whether the stands in some localities are natural or artificial (4). *P. pinea* does well in the warmer regions of the United States.

In 1917 Palazzo (9) fractionated a batch of *P. pinea* commercial turpentine; it began to boil at 162–163°, and 75 or 80 per cent of it distilled between 163 and 178°. Possibly the head fraction contained minute quantities of α-pinene. Under laboratory conditions, using steam at 2 to 3 atmospheres of pressure, Palazzo obtained turpentine with the characteristics (average of eight samples) shown in Table I. Only 1.04 per cent of the turpentine distilled below 173°, and 90 per cent of it distilled below 180°. No α-pinene was reported; the oil apparently consisted almost entirely of *l*-limonene. The sesquiterpene fraction was very small (probably less than 5 per cent) and it was not investigated.

Lacrué (7) also reported characteristics of *P. pinea* turpentine (Table I) which indicate the presence of large amounts of *l*-limonene.

Dupont and Barraud (5) analyzed *Pinus pinea* commercial turpentine, obtained from the province of Granada, Spain. Yield of (steam distilled) turpentine was 16 to 18 per cent; its characteristics are given in Table I. Purified *l*-limonene, obtained by fractional distillation of the crude turpentine possessed specific rotation $[\alpha]_j$ equal to −123.7°. The composition of the turpentine was as follows: *l*-α-pinene, 16.7% *l*-limonene, 75.4%; and sesquiterpene, 6.6%. Lacrué (7) questioned the presence of α-pinene in turpentine of *P. pinea*. He said the sample analyzed by Dupont and Barraud contained an admixture of *P. maritima* turpentine which is rich in pinene. The sesquiterpene was optically inactive, having density, $d^{16} = 0.9157$ and index of refraction, $n_D^{20} = 1.4983$. No crystalline derivatives (nitrosochloride, nitrosite, nitrosate, bromide) were obtained.

Pinus pinea turpentine was obtained in Turkey by Berkel (3) (Table I). Initial boiling point of Berkel's turpentine at 760 mm. was 172–173°. Only 0.09% distilled above 181°. About 90% distilled between 175–177°, and the specific rotation of this fraction was: $[\alpha]_D^{20} = -118.7$ to −119.3°. Hence there was very little, if any, α-pinene and very little sesquiterpene.

To sum up, past investigations have shown that *Pinus pinea* turpentine, obtained throughout its natural range, consists almost entirely of *l*-limonene; α-pinene apparently is either completely lacking or is found in minute quantities.

Oleoresin for the present studies was obtained in the summer of 1953[1] from five 25-year-old planted trees at the Institute of Forest Genetics, Placerville, Calif. The trees were grown from seeds received from Italy.

EXPERIMENTAL

A batch of 1,665 Gm. of oleoresin was heated under reduced pressure so that at the end of the process the temperature was 195° and the pressure was 0.5 mm. of mercury. Yield of turpentine was 18.5%. Physical characteristics of the crude turpentine are shown in Table I.

A batch of 300 Gm. of the turpentine was fractionated in a 90-cm. long, 25-mm. inside diameter Todd column packed with glass helices. A 10:1 reflux ratio was maintained during fractionation. The results are shown in Table IV.

Nitrosochloride prepared from fraction 1 melted at 110°. Piperidide was prepared from the nitrosochloride, but the melting point was not 118–119° as it should have been for pinene piperidide, but 107.5°. When the nitrosochloride was mixed with an authentic pinene nitrosochloride, having melting

* Received October 11, 1954, from the Institute of Forest Genetics, California Forest and Range Experiment Station maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

† The work reported in this paper was aided through a grant from the Rockefeller Foundation.

[1] Acknowledgment is given Mr. Emery Kimbrough for assisting in collection of the oleoresin sample.

point of 107–108°, the melting point was depressed to 103°. Accordingly, it appears that fraction 1 contains very little, if any, α-pinene.

Judging from the physical characters, fractions 1 to 6 are composed almost entirely of *l*-limonene. Its presence was verified by preparing limonene tetrabromide from fraction 5 which, after several recrystallizations from ethyl ether, melted at 105°.

A 2-Gm. sample of fraction 8 was dissolved in eight volumes of absolute ether. Dry HCl gas was passed through the solution, which was kept between 0 and 5° by means of an ice-salt bath. The treated oil was kept in a stoppered flask in the refrigerator for one week; then the ether was allowed to evaporate, and the oily residue was kept in a watch glass in the refrigerator for three more days. The solidified oil contained some crystals; these were pressed out on a porous plate. After three recrystallizations from glacial acetic acid, the hydrochloride melted at 67.5–68.5°.

Pinus torreyana (6) of the group *Macrocarpae* and with *Pinus pinceana* of the group *Cembroides*. The latter relationship is also supported by the presence of apparently the same sesquiterpene in the two pines.

PINUS HALEPENSIS

Pinus halepensis Miller grows in parts of southern Europe, Asia Minor, and northern Africa, adjacent to the Mediterranean Sea.

Turpentine of this pine obtained in France (11), Spain, Algeria, Italy, and Greece (7) was found to be dextrorotatory (+42.83 to +48.52°); it consisted chiefly of *d*-α-pinene (up to 95%) and of about 4% of sesquiterpenes. One per cent of bornyl acetate was reported by Vèzes and Dupont (11). Older data giving specific rotation values for *P. halepensis* of −8.73, +2.84, +1.61, and +0.28 are considered by Lacrué as not being correct because these data refer to commercial mixtures consisting of dextrorotatory *P. halepensis* turpentine and levorotatory *P. laricio* turpentine.

TABLE I.—PHYSICAL CHARACTERISTICS OF P. PINEA TURPENTINE

Author	Origin of the Turpentine	Density, d	Index of Refraction, n_D	Specific Rotation $[\alpha]_D$
Palazzo (9)	Italy	0.8473^{15}–0.8514^{15}	1.4731–1.4751^{20}	−93.80 to −97.30°
Dupont (5)	Spain	0.8506^{15}	1.4700^{25}	−84.53°
Audrain[a]	Spain	0.8513^{17}	1.4769^{15}	−82.62°
Lacrué (7)	Spain	0.8435–0.8498^{15}	1.4750–1.4775^{17}	−92.68 to −96.69°
Berkel (3)	Turkey	0.8400–0.8440^{20}	1.4740–1.4770^{20}	−118.7 to −119.3[b]
Present experiment	California	0.8393^{24}_{4}	1.4723^{23}	−118.0°

[a] Quoted by Lacrué (7).
[b] Of the main fraction. See text.

There are no indications in the literature on occurrence of a sesquiterpene hydrochloride possessing such melting point, except of a hydrochloride obtained in our laboratory from *Pinus pinceana* sesquiterpene (8). The physical characteristics of the two sesquiterpenes are given in Table II.

EXPERIMENTAL

A sample of oleoresin used in the present experiment came through the courtesy of Chief Forester, State of Israel, Dr. A. Y. Goor. It was collected on the slopes of historic Mt. Carmel, and shipped to our laboratory in Berkeley, Calif. A batch of the oleoresin weighing 3,063 Gm. was distilled *in vacuo*, so that at the end of the distillation the temperature was increased to 180° and the pressure reduced to 0.1 mm. of mercury. The yield of turpentine was 20% of the weight of the oleoresin. The turpentine had the following characteristics: $d^{23}_{4} = 0.8581$; $n^{24}_{D} = 1.4663$; $[\alpha]^{23}_{D} = +41.2°$.

A batch of 606 Gm. of the turpentine was distilled in the previously mentioned Todd column, using a

TABLE. II—PHYSICAL CHARACTERISTICS OF SESQUITERPENES FROM TURPENTINE OF P. PINCEANA AND P. PINEA

Species	Density, d	Index of refraction, n	Optical rotation, $[\alpha]$	Boiling range, °C.	Molecular refraction, MR^{23}_{D}	Melting point of hydrochloride, °C.
P. pinceana (8)	0.9117^{23}_{4}	1.500^{23}_{D}	Slightly negative	259–60 at 760 mm.	65.8	67–68
P. pinea (5)	0.9157^{15}	1.4965^{25}_{D}	Inactive	128.5–9 at 13 mm.	65.2	Not prepared
P. pinea, present experiment	0.9037^{23}_{4}	1.4993^{23}_{D}	Inactive	110° at 4 mm.	66.3	67.5–68.5

To sum up, turpentine of *Pinus pinea* obtained from planted trees in California consists of *l*-limonene, 95%, and a bicyclic sesquiterpene having 2 double bonds, 4%. This sesquiterpene is apparently the same as in the turpentine of *Pinus pinceana* of Mexico. Presence of α-pinene is doubtful. It appears that turpentine from *P. pinea* grown in its Mediterranean home and in California has the same composition, i. e., it consists mostly of *l*-limonene. This seems to indicate that with change of environment, chemical composition of turpentine does not change. Presence of large quantities of limonene suggest relationship of this pine with

TABLE III.—PHYSICAL PROPERTIES OF TERPINOLENE AND FRACTION 14 of P. BRUTIA TURPENTINE

Property	Fraction 14	Terpinolene[a]
d_4	0.8600^{22}	0.8560^{25}
n_D	1.4851^{22}	1.4864^{25}
$[\alpha]_D$	0.0	0.0

[a] Sutherland, M. D., Univ. of Queensland Papers, Vol. 1, No. 34 (1948).

10:1 reflux ratio. The results of the distillation are shown in Table IV.

Fractions 1 and 2 had the odor and physical characteristics of *d* and *dl*-α-pinene. As previous investigators have found this pine to contain α-pinene (11), no further work was done with these fractions.

Fraction 4 had an unusually low density which indicated the possible presence of β-myrcene. A portion of this fraction was converted to a maleic anhydride adduct which, after several recrystallizations from hexane at 0°, melted at 33–34° and showed no melting point depression on admixture with authentic maleic anhydride adduct of β-myrcene.

TABLE IV.—FRACTIONAL DISTILLATION OF TURPENTINES OF P. PINEA, P. HALEPENSIS, AND P. BRÚTIA

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density $d_4^{22.5}$	Index of Refraction n_D^{22}	Specific Rotation $[\alpha]_D^{22}$
		Pinus pinea (300 Gm. used)				
1	760	165–174	2.0	0.8384	1.4703	−102.5
2	760	174–175	8.5	0.8375	1.4712	−112.0
3	760	175–176	16.5	0.8375	1.4719	−117.3
4	760	176–176.5	27.1	0.8372	1.4719	−123.5
5	760	176.5–176.6	34.2	0.8389	1.4723	−125.2
6	760	176.6–176.7	6.7	0.8408	1.4729	−124.3
7	14	90–120	0.7	0.8782	1.4839	−62.6
8	14	120–130	3.3	0.9044	1.4995	−3.0
Residue and losses			1.0			
				d_4^{25}	n_D^{22}	$[\alpha]_D^{22}$
		Pinus halepensis (606 Gm. used)				
1	13	40–41	74.4	0.8536	1.4650	+49.6
2	13	41	11.1	0.8536	1.4650	+49.1
3	13	41–49	1.2	0.8536	1.4684	+40.1
4	13	49–60	1.2	0.8254	1.4719	+12.8
5	13	60–61.5	0.3	..	1.4719	+12.0
6	13	61.5–62	0.4	0.8489	1.4741	+14.9
7	13	62–100	0.1	..	1.4772	...
8	13	100–105	0.2	0.9091	1.4809	...
9	13	105–110	0.3	0.9203	1.4862	+14.4
10	13	110–115	0.6	0.9133	1.4905	+5.0
11	13	115	0.4	0.9009	1.4945	−4.5
	Pot contents transferred to a Claisen flask and distillation continued.					
12	7	105–110	0.7	0.8988	1.4970	−7.1
13	6	105	1.2	0.8993	1.4987	−9.1
14	2	110	1.0	0.9106	1.5015	−28.1
Residue and losses			6.9			
				d_4^{25}	n_D^{22}	$[\alpha]_D^{22}$
		Pinus brutia (457 Gm. used)				
1	19	43–44.5	19.0	0.855	1.4646	−44.9
2	19	44.5–46	37.0	0.855	1.4651	−44.9
3	19	46–49	4.7	0.858	1.4697	−36.6
4	19	49–51	4.0	0.863	1.4745	−27.3
5	19	51–52	6.0	0.865	1.4760	−21.1
6	19	52–54	4.0	0.865	1.4764	−13.9
7	19	54–56	3.1	0.861	1.4744	−4.3
8	19	56	1.4	0.856	1.4729	+5.4
9	18	55–55.5	8.4	0.859	1.4721	+13.3
10	18	55.5–60	2.1	0.873	1.4755	+11.4
11	18	60–62	0.8	0.856	1.4750	+7.0
12	18	62	0.6	0.854	1.4777	+3.0
13	18	62–70	0.9	0.854	1.4818	0.0
14	12	69	0.8	0.860	1.4851	0.0
15	12	69–78	0.4	0.885	1.4882	−1.4
16	12	78–103	0.6	0.927	1.4895	−14.2
17	12	103–117	0.4	0.948	1.4949	−14.4
18	12	117–119	0.6	0.937	1.4974	+7.0
19	12	119–121	1.0	0.918	1.4999	+12.0
20	12	121	2.0	0.912	1.5007	+12.1
21	12	121	0.8	0.909	1.5009	+10.5
Residue and losses			1.4			

Fractions 5 and 6 were mostly β-myrcene. Fractions 7, 8, and 9 were small transition fractions containing some oxygenated compounds, perhaps some bornyl acetate.

Fractions 10 to 14 contained sesquiterpenes. It was attempted to prepare a hydrochloride from fraction 10 and fraction 14 but no derivative was obtained when these fractions were treated in the usual manner for hydrochloride preparation.

To sum up, the turpentine of *P. halepensis* from Israel contained 87% *d*- and *dl*-α-pinene: 2% β-myrcene; 4% sesquiterpenes; and 7% pot residue and loss.

PINUS BRUTIA

Pinus brutia Ten. has a more restricted range than *P. halepensis*. It grows in the Italian province of Calabria (ancient Brutium), in Syria, Turkey, Greece, and on the islands of Chios, Rhodes, Crete, and Cyprus. Some botanists consider *P. brutia* as a variety of *P. halepensis* (4), others even believe that the two names are synonymous (10). *P. brutia* is immune to *Matsucoccus* infestation, while *P. halepensis* is susceptible to the attacks of this scale insect.

Very little is known about the chemical composition of *P. brutia* turpentine. An anonymous author (1) described commercial turpentine obtained from "*P. halepensis*" from Cyprus (i. e., of *P. brutia*, for *P. halepensis typica* does not occur on that island). The turpentine was found to be levorotatory (−20.2; −32.5). Only α-pinene was identified; it amounted to 55% of the total turpentine. Fractions boiling above 160° were not investigated. A closely related pine, *P. pithyusa*, of the Caucasus was found by Arbuzov (2) to consist of about 70% *l*-α-pinene, about 24% Δ^3-carene, and 5 to 6% of higher boiling, unidentified components.

A sample of *P. brutia* came to us through the courtesy of Mr. D. F. Davidson, Conservator of Forests, Government of Cyprus. It was collected in the Dhiorios Forest, Cyprus. The oleoresin was distilled under the same conditions as that of *P. halepensis*, (i. e., 0.1 mm./180°). Yield of turpentine was 20%.

The crude turpentine had the following physical characteristics: $d_4^{24} = 0.8571$; $n_D^{25} = 1.4612$; $[\alpha]_D^{24} = -28.7°$.

EXPERIMENTAL

A batch of 457 Gm. of the turpentine was distilled through the previously mentioned column. A reflux ratio of 10:1 was maintained to fraction 9 after which a 5:1 reflux ratio was used. A total of 457 Gm. of distillate was received. The pot residue was negligible. The results of the distillation are shown in Table IV.

The properties of fractions 1 and 2 are similar to the properties of α-pinene. *dl*-α-Pinene nitrosochloride was prepared from fraction one. The nitrosochloride, after two recrystallizations from chloroform by addition of cold methanol, melted at 103.5–104° and was not depressed in m. p. by an authentic sample of *dl*-α-pinene nitrosochloride.

Fraction 3 is a transition fraction consisting of a mixture of α- and β-pinene.

Fractions 4, 5, and 6 have properties indicating that they are mainly *l*-β-pinene.

Ten Gm. of fraction 5 was oxidized with alkaline potassium permanganate solution (aq.) using the method of Wallach. The sodium nopinate received was converted into nopinic acid. The nopinic acid was recrystallized twice from benzene, after which 2.1 Gm. of the acid, m. p. 127°, was received. The nopinic acid was not depressed in melting point by addition of an authentic nopinic acid.

Fractions 7 and 8 are transition fractions containing both *l*-β-pinene and *d*-Δ^3-carene. *d*-Δ^3-Carene nitrosate was prepared from fraction 9. After recrystallization from chloroform by addition of cold methanol, the nitrosate melted at 146° and was not depressed in melting point by an authentic sample of the *d*-Δ^3-carene nitrosate.

Fractions 11, 12, and 13 are transition fractions containing Δ^3-carene and terpinolene. Fraction 14 has physical properties similar to terpinolene (Table III). From 2 ml. of fraction 14, about 1.48 Gm. of tetrabromide was prepared. The tetrabromide was recrystallized once from chloroform by addition of cold methanol and then melted at 117–118°; it showed no depression in melting point on admixture with authentic terpinolene tetrabromide.

Fractions 15 and 16 are transition fractions.

The fractions from 17 to 21 contain sesquiterpenes.

To sum up, the gum turpentine of *P. brutia* is composed of: *l*- and *dl*-α-pinene, 62%; *l*-β-pinene, 17%; *d*-Δ^3-carene, 13%; terpinolene, 2%; sesquiterpenes, 4.6%; pot residue and loss, 1.4%.

Chemical composition of the turpentine of *Pinus brutia* differs so much from that of *Pinus halepensis* that we are inclined to consider the two pines as separate species.

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CORRECTION

In the December, 1954 issue of THIS JOURNAL, an error occurred in "Composition of Gum Turpentines of Pines, XXI" by P. M. Hoff and N. T. Mirov. Paragraph 7, left column, p. 740 through paragraph 5, right column should appear at the end of the text, before references on p. 741.

Book Notices

British Pharmaceutical Codex 1954. Published by direction of the Council of the Pharmaceutical Society of Great Britain. xxxii + 1340 pages (including Index). 23 x 16 cm. The Pharmaceutical Press, London, 1954. Price 63*s*.

The first edition of the British Pharmaceutical Codex, which was published by the Council of the Pharmaceutical Society of Great Britain in 1907, was conceived as a reference book for those engaged in prescribing or dispensing medicines. The new sixth edition seeks to maintain the original intent of the Council within the limitations of reasonable space. The great increase in the number of therapeutically acceptable drugs has required certain revisions in the scope of the revision committee.

It is no longer feasible to include monographs on all the drugs that are still in use. The inclusion of a drug in the Codex is now regarded as an indication that it is of value or is an established constituent of a useful preparation. However, even with the period between editions reduced to five years, which other drug compendia in Great Britain and the United States have found to be necessary, the problems of selection for admission or deletion are numerous.

Although the force of law may not be definitely assigned by the British Food and Drugs Act of 1938 to the standards for drugs which are admitted to the Codex but are not in the British Pharmacopoeia, these standards have proved valuable to the Courts of Law in making decisions on the quality of a drug and to the authorities administering testing schemes under the National Health Service.

The use of the Codex as a book of reference primarily demands the inclusion of such information as action and uses of drugs. Drugs that are widely prescribed although convincing clinical evidence of their value is lacking are included unless there is acceptable evidence that they are ineffective; but tradition or sentiment has not been considered an adequate reason for inclusion. On the other hand an effective drug that might be considered undesirable for use for certain reasons might still be included such as the monographs for preparations of Diamorphine Hydrochloride. However, the monograph for the salt includes a recommendation that the drug should be used with great caution and only when less dangerous cough depressants have proved inadequate or unsuitable.

The British have now made English the primary language of nomenclature in the Codex as was done in the B. P. 1953. The Latin names are retained as subsidiary titles or synonyms. Although the U. S. P. and N. F. system of marginal arrangement of titles for rapid reference is not used, the monographs are arranged alphabetically according to the important part of the title. Thus, between Ipecacuanha and Ipomoea appears Prepared Ipecacuanha; Sodium Phosphate is followed by Exsiccated Sodium Phosphate then Sodium Potassium Tartrate; and Oxytetracycline Hydrochloride is followed by Injection of Oxytocin then Pamaquin. In most instances this also follows the order that would be maintained by the Latin form of the titles.

The main text of the Codex is divided into six parts: General Monographs (mainly individual drugs, but some dosage forms); Antisera, Vaccines and Related Substances; Preparations of Human Blood; Surgical Ligatures and Sutures; Surgical Dressings; and Formulary (dosage forms). In the Formulary Section, Part VI, are the dosage forms of the drugs described in the General Monographs Section, Part I. "Standards, including tolerances and methods of assay have been included for many preparations which, although usually made extemporaneously, are like to be taken as samples under testing schemes. In deciding suitable tolerances, account has been taken of the quantities usually prepared, factors in dispensing which may lead to variation, and any inherent errors due to methods of assay and of sampling."

Eye-Drops must be freshly prepared with aseptic precautions, a suitable fungistatic agent should be used in preparations liable to support the growth of molds, and they should be dispensed in sterilized containers. Included also is the statement: "Care should be taken to avoid contamination during use." It is not clear whether this is a suggestion that an antibacterial agent in addition to the fungistatic agent should be added, or whether the pharmacist is expected to explain the proper use of the product to the purchaser and perhaps supply him with the correct number of sterile droppers or applicators. Of the twenty-seven formulas given under Eye-Drops, only one monograph, Oily Eye-Drops of Cocaine and Mercuric Chloride, includes standards and an assay method.

As in most compendia revisions in this era of rapidly changing materia medica, extensive changes from the 1949 Codex have been made. More than 250 basic drug monographs and nearly 400 formulas for dosage forms were not readmitted in this revision. Many botanical drugs and essential oils which were rarely used as drugs were dropped, and groups of products such as the iron salts and quinine salts have been reduced in a manner that more nearly reflects their use and usefulness. Newly admitted monographs are concerned mainly with synthetic drugs. Among the approximately 70 basic drugs, four are antihistamines, four antimalarial agents, two new sulfonamides, two anticoagulants, topical fungicides, cationic detergents, antituberculous agents, and four salts of the ganglion-blocking agent hexamethonium. About 84 dosage forms have been added in this revision of the Codex.

The arrangement of the monographs is similar to that in the 1949 Codex, and the tests and standards are of the high calibre one has come to expect in this compendium. The Appendices provide: tables of equivalents of weights and measures, information on reagents, quantitative tests for arsenic and lead, tabulated alcohol limits for dosage forms with the indicated B. P. assay method, and other general information. Chapters on isotonic solutions and sterilization are also included in the Appendices Section.

The excellence of the results achieved by the

groups responsible for selection and development of the text material in the British Pharmaceutical Codex 1954 make it a reference volume of value not only to pharmacists throughout the British Commonwealth but to all who are concerned with the professional practice of pharmacy everywhere, and the book should be available to students through their libraries.

Reproductive System. By FRANK H. NETTER. Ciba Pharmaceutical Products, Inc., Summit, New Jersey, 1954. xvi + 286 pp. 24 x 31.5 cm. Price $13.

This new addition to Ciba's series of medical illustrations is a worthy follower of the first title which was given over to the nervous system. The volume does not only present some of the best illustrations of reproductive anatomy, physiology, and pathology, but it is also enhanced by a text well suited to point up and enlighten our ignorance of reproductive processes.

Jahrhundertfeier der Geburtstage von Paul Ehrlich und Emil V. Behring. Farbwerke Hoechst AG., Frankfurt (M)-Hoechst., 1954. Illustrated. 27.5 x 20.3 cm.

The Farbwerke Hoechst have published a most attractive book of photographs to serve as a permanent souvenir of the centennial celebration held at Frankfurt a. M. and Marburg, in honor of Paul Ehrlich and Emil V. Behring. This pictorial report should give an impressive idea of the character and harmony of the festivities to all those who could not attend them. Particularly touching are the portraits of Dr. Ehrlich's old secretary and biographer, Fräulein Martha Marquardt.

Practical Clinical Biochemistry. By HAROLD VARLEY. Interscience Publishers, Inc., New York, 1954. vii + 551 pp. 14.5 x 17.3 cm. Price $6.50.

This book presents a survey of the methods employed in the entire field of clinical biochemistry and their importance in medical practice. The subject matter is designed to appeal particularly to workers in hospital laboratories, such as those in the field of clinical pathology and biochemistry. The book should also be useful to technicians in clinical laboratories. It is also provided with several useful appendices and an entirely adequate subject index.

The Vitamins—Chemistry, Physiology, Pathology. Vol. 3, W. H. SEBRELL, JR., and ROBERT S. HARRIS, Editors. Academic Press, Inc., New York, 1954. xv + 665 pp. 16 x 23.5 cm. Price $15.

Volume III of *The Vitamins* constitutes the last in a series of three. The announcements concerning the first two volumes can be found in THIS JOURNAL, **43,** 448(1953), and **44,** 63(1954). As previously mentioned, these volumes are designed to serve mainly as a reference work, and the chapters are divided into several parts, each of which has been contributed by an outstanding authority on the aspects of each of the vitamins considered. An attempt has been made, apparently successful, to present an outline of the current knowledge concerning the chemistry, industrial preparation, physiology, biochemistry, estimation, occurrence, deficiency effects, pharmacology, and the requirements of each vitamin. No attempt has been made to present in detail the clinical manifestations of vitamin deficiency and their treatment because these are adequately covered in other publications. Similarly, methods of vitamin assay are discussed only briefly.

In Volume 3, current information is presented in seven chapters entitled *p*-Aminobenzoic Acid, Pteroylglutamic Acid, Pyridoxine and Related Compounds, Riboflavin, Thiamine, the Tocopherols, and New and Unidentified Growth Factors With the completion of three volumes of *The Vitamins* the contributors and editors have made an outstanding contribution by summarizing so concisely all pertinent information relating to the field covered. All three volumes are well documented throughout, and each is provided with an author and subject index. The printing and binding are of excellent quality and the editors, publishers, and contributors are to be congratulated upon making this new series of books on the vitamins available to investigators and practitioners.

The Plant Quarantine Problem. By W. A. MCCUBBIN. Ejnar Munksgaard, Copenhagen (The Chronica Botanica Co., Waltham, Mass.). 255 pp. 15 x 22.5 cm.

The efforts of various countries to prevent the impairment of their agricultural resources through the introduction of destructive pests from other countries have gradually developed into highly technical administrative systems. An expert discussion of these systems of a plant quarantine had not been previously available so that this book fills a very definite need. The author, formerly for twenty years an advisor on plant disease problems for the U. S. Bureau of Entomology and Plant Quarantine, deals with the varied aspects of his problem in chapters entitled: The Biological Background; Social and Economic Relations; Legal Features; Administrative Aspects; A Current Quarantine Problem Examined; Appraisal and Outlook; and The World Situation. The examination of an actual quarantine problem is presented, showing the intricacies involved in any quarantine procedure, which adds to the interest of the book, but to the average reader, the historical summary of federal plant disease quarantines which is given as an appendix will be its most attractive feature. It shows what has been done to protect our shade and fruit trees, our citrus orchards, and our potato supply, and thus points up the general importance of the author's chosen topic. This well-written book deserves to be widely read.

Hypertension.—Humoral and Neurogenic Factors. (Ciba Foundation Symposium). G. E. W. WOLSTENHOLME and MARGARET P. CAMERON, Editors. Little, Brown and Company, Boston, 1954. xvi + 294 pp. 21 x 14.5 cm. Price $6.75.

This volume affords the reader an opportunity to join a group of outstanding investigators into the causative factors and related symptoms in certain

forms of hypertension. The informal discussions that follow the papers presented at the symposium are most enlightening. Chairman G. W. Pickering opened the symposium with a brief speech which included the statement: "It is not surprising, if we know so little about the mechanism of hypertension in the animal, that we know even less about it in man. . . ." Attempts to increase that knowledge are represented in the papers presented under the following titles: Neural and Humoral Control of Blood Vessels; Some New Aspects of Reflex Blood Pressure Regulation and Hypertension; Hypertensive Disease without Hypertension; Internal Secretion of the Arterial Wall in Blood Pressure Regulation; Drugs Antagonistic to 5-Hydroxytryptamine; Quantitative Estimation of 5-Hydroxytryptamine in Gastro-Intestinal Mucosa, Spleen and Blood of Vertebrates; Chemical Screening Methods for the Diagnosis of Phaeochromocytoma; Persistence of Hypertension after Removal of Phaeochromocytoma, where Excretion of Adrenaline and Noradrenaline is Normal; Experimental Studies on the Pathogenesis and Nature of Hypertensive Cardiovascular Disease; Experiments on the Role of Vasoconstrictor Substances in the Mechanism of Renal Hypertension in Dogs; The Relation of the Sustained Pressor Principle to Renin; The Role of the Kidney in the Mechanism of Experimental Hypertension; The Effect of Partial Renal Corticectomy on the Kidneys of Normal and Hypertensive Animals; The Use of Ganglion Blocking Agents in Relation to Neurogenic Factors in Hypertension; Lessons from Hexamethonium Studies in Malignant Hypertension; Catechol Amine Excretion in Urine in Cases of Hypertension; The Relationship Between Sodium, Arterial Hypertension and the Adrenal Glands; The Study of Adrenal Insufficiency in Hypertensive Patients Submitted to Bilateral Sympathectomy and Unilateral Adrenalectomy; Water and Electrolytes in Experimental Hypertension; The Distribution of Fluid and Electrolytes in Experimental Hypertension; and The Importance of Sodium Chloride and Its Ions in the Production and Treatment of Hypertension.

This book should be helpful to those who are engaged in the study of hypertension, and it should be available to everyone who is interested in the latest developments and indicated future lines of experimental study in this field.

BOOKS RECEIVED

Einfuhrung in die Biologische Registriertechnik. By Dr. Med. HERBERT KLENSCH. Georg Thieme Verlag, Stuttgart, 1954 (Intercontinental Medical Book Corporation, New York 16, N. Y.). x + 222 pp. 24.75 x 17.75 cm. Price $4.85.

Les Enrobages Modernes des Dragées et des Pilules. By ALBERT COUVREUR. Vigot Frères, Paris, 1954. 24 x 16 cm. 130 pp.

Glutathione. Proceedings of the Symposium held at Ridgefield, Connecticut, November, 1953. Academic Press, Inc., New York, 1954. x + 341 pp. 15.5 x 23.5 cm. Price $7.50.

Hospital Formulary of Selected Drugs. By DON E. FRANCKE. Hamilton Press, Hamilton, Ill. xvi + 759 pp. 12.5 x 19 cm. Price $5.

Jahrhundertfeier der Geburtstage von Paul Ehrlich und Emil V. Behring. Farbwerke Hoechst Ag., Frankfurt (M)-Hoechst., 1954. Illustrated. 27.5 x 20.3 cm.

Organic Reactions. Vol. 8. ROGER ADAMS, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1954. viii + 437 pp. 15.5 x 23.5 cm. Price $12.

Practical Clinical Biochemistry. By HAROLD VARLEY. Interscience Publishers, Inc., New York, 1954. vii + 551 pp. 14.5 x 17.3 cm. Price $6.50.

Somministrazione dei Farmaci per via Rettale Tecnica di Preparazione delle Forme Farmaceutiche. By G. GIACOMINI and E. MASCITELLI. Gitti Editore, Milan, 1954. 341 pp. 24 x 17 cm. Price L 3500.

Actualities Pharmacologiques. By J. M. BURN, J. CELICE, J. CHEYMOL, R. DOMENJOZ, R. HAZARD, J. JACOB, Ch. MENTZER, F. G. VALDECASAS. Masson et Cie, Editeurs, Paris-6, France. (Published under the direction of Rene Hazard, 1954. 210 pp. 17 x 25 cm. Price 1480 fr.

Calendar of the Pharmaceutical Society of Great Britain 1954/55 The Pharmaceutical Press, London, 1954. vi + 306 pp. 22 x 14.5 cm. Price 12*s.* 6*d.* (Postage 10*d*).

50 Jahre Arzneimittelforschung. By C. L. LAUTENSCHLAGER. Georg Thieme Verlag, Stuttgart, 1955. (Intercontinental Medical Book Corporation, New York). 486 pp. 25 x 18 cm. Price $8.60.

The Kidney. (A Ciba Foundation Symposium). A. A. G. LEWIS and G. E. W. WOLSTENHOLME, Editors. Little, Brown and Company, Boston, 1954. xvi + 333 pp. 21 x 14.5 cm. Price $6.75.

The Lipids.—Their Chemistry and Biochemistry. Vol. 2, Biochemistry (Digestion, Absorption, Transport and Storage). By HARRY J. DEUEL, JR. Interscience Publishers, Inc., New York, 1955. xxvi + 919 pp. 24 x 16.5 cm. Price $25.

Perspectives and Horizons in Microbiology. A Symposium. Edited by SELMAN A. WAKSMAN. Rutgers University Press, New Brunswick, N. J., 1955. x + 220 pp. 22 x 14.5 cm. Price $3.50.

Pharmacodynamie Biochimique. By Z. M. BACQ, J. CHEYMOL, M. J. DALLEMAGNE, R. HAZARD, J. la BARRE, J.-J. REUSE, and M. WELSCH. Masson et Cie, Editeurs, Paris-6, France, 1954. xvii + 1008 pp. 17.5 x 26 cm. 6.000 fr.

Allgemeine chemische Methoden. Vol. 4. Part 2. B. HELFERICH, H. HENECKA, G. HESSE, H. KOCH, J. KREBS, H. KRÖPER, F. MÜLLER, W. REISNER, G. SCHILLER, H. SIMON, K. STETTER, W. THEILACKER, F. WEYGAND, K. WIMMER, K. ZIEGLER, Editors. Georg Thieme Verlag, Stuttgart, 1955. XXVIII + 1004 pp. 26 x 19 cm. Price $36.20.

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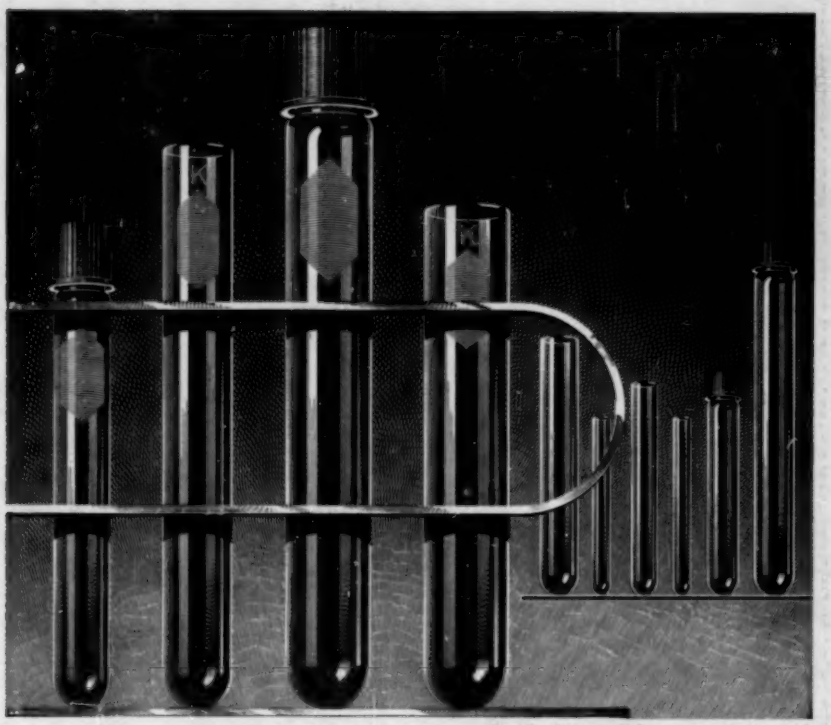

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